DESCRIPTION

YEAST-DERIVED PROMOTERS, AND THE VECTORS AND EXPRESSION SYSTEMS USING THE PROMOTERS

Technical Field

The present invention relates to a DNA fragment having a cold-inducible promoter function of yeast.

Background Art

Yeast has widely been used for production of foods by fermentation, such as alcoholic beverages including beer or Japanese sake, or breads, for production of metabolites such as amino acids, and also as a host used for production of proteins of homogeneous or heterogenous organisms using the recombinant DNA technique. The characteristics of yeast used in production of proteins by such recombinant DNA technology include: the safety of yeast as an organism, which is assumed from the past record in that yeast has previously been used in the food industry; a relatively high probability of success in the expression of proteins of animals such as a human because yeast is not a prokaryote such as *Escherichia coli*, but a eukaryote; and sufficiently developed gene recombination technology regarding yeast.

In general, it has been already known regarding production of beer or brewage that fermentation at a low temperature such as 10°C or lower brings on exquisite flavor and taste, and that the quality as food can be improved. Since the existence of a chemical substance for improving flavor or taste is assumed from such improvement of flavor and taste, it is considered that the functions of a gene of an enzyme synthesizing such a chemical substance are appropriately regulated by decreasing the temperature. However, there is only a limited amount of information regarding genes of yeast functioning at a low temperature. Thus, the type of a gene that is important for improvement of the flavor or taste of foods is still unknown.

In gene recombination technology using yeast or *Escherichia coli* as a host, promoters functioning at an ordinary culture temperature (30°C in the case of yeast and 37°C in the case of *Escherichia coli*) have conventionally been used to produce proteins. In general, strong promoters producing a more large amount of mRNA have been used. It is considered that culture at a low temperature is disadvantageous in the production of proteins by genetic recombination. As a matter of fact, however, there are some cases where a low temperature is intentionally used to produce proteins. For example, when a protein produced at an ordinary temperature does not have a correct three-dimensional structure, a protein having a correct three-dimensional structure may be then produced at a low temperature. Thus, in order that a protein has a correct three-dimensional structure, there are some cases where production of a protein may be carried out at a culture temperature that is 10°C lower than the ordinary temperature (Prot. Exp. Purif. 2, 432-441 (1991)). In addition, it is also expected that application of such a low temperature prevent the produced protein from being decomposed with protease of a host. Thus, it is considered that production of proteins at a low temperature has advantages. On the other hand, it is also considered that in the case of the currently used promoter functioning at an ordinary temperature, the promoter activity decreases together with a decrease in the temperature. Accordingly, it is appropriate to use a promoter exhibiting high activity in a low temperature range to establish an efficient protein production system at a low temperature.

To date, there has been a report that the mRNA of each of YBR067C (TIP1), YER011W (TIR1), YGR159C (NSR1), YGL055W (OLE1), YOR010C (TIR2), YKL060C (FBA1), YIL018W (RPL2B), YDL014W (NOP1), YKL183W, YKL011W, and YDR299W (BFR2), is increased by treating the yeast at a low temperature. However, the degree of cold inducibility of each of the promoters of the above genes has not yet been examined.

Disclosure of the Invention

It is an object of the present invention to provide, for example, a DNA fragment having a cold-inducible promoter function of yeast, which has high activity in a low temperature

range (e.g. 10°C or lower), by identifying and analyzing a large number of cold-inducible genes of yeast.

As a result of intensive studies directed towards achieving the aforementioned object, the present inventors have identified genes of *Saccharomyces cerevisiae* exhibiting cold inducibility using a DNA microarray, and have found a DNA fragment having a cold-inducible promoter function in the non-translation region located upstream of the 5'-terminal side of each gene, thereby completing the present invention.

That is to say, the present invention relates to a DNA fragment, which exists in the non-translation region located upstream of the 5'-terminal side of a gene selected from the group consisting of genes of *Saccharomyces cerevisiae* described in Table 1 indicated below, and has a cold-inducible promoter function.

Table 1

No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name
1	YAL014C	53	YDR184C	105	YGR008C	157	YKL224C	209	YNR071C
2	YAL015C	54	YDR219C	106	YGR043C	158	YKR049C	210	YNR075W
3	YAL025C	55	YDR253C	107	YGR053C	159	YKR075C	211	YNR076W
4	YAL034C	56	YDR256C	108	YGR088W	160	YKR077W	212	YOL002C
5	YBL048W	57	YDR262W	109	YGR102C	161	YKR100C	213	YOL016C
6	YBL049W	58	YDR306C	110	YGR154C	162	YLL055W	214	YOL084W
7	YBL054W	59	YDR336W	111	YGR197C	163	YLL056C	215	YOL101C
8	YBL056W	60	YDR346C	112	YGR222W	164	YLR009W	216	YOL108C
9	YBL065W	61	YDR387C	113	YGR223C	165	YLR145W	217	YOL116W
10	YBL078C	62	YDR398W	114	YGR251W	166	YLR149C	218	YOL124C
11	YBR016W	63	YDR435C	115	YGR256W	167	YLR164W	219	YOL127W
12	YBR018C	64	YDR453C	116	YGR262C	168	YLR251W	220	YOL132W
13	YBR024W	65	YDR471W	117	YGR286C	169	YLR252W	221	YOL153C
14	YBR034C	66	YDR492W	118	YGR294W	170	YLR266C	222	YOL154W
15	YBR045C	67	YDR496C	119	YHL016C	171	YLR311C	223	YOL161C
16	YBR047W	68	YDR504C	120	YHL021C	172	YLR312C	224	YOL162W
17	YBR050C	69	YDR516C	121	YHL036W	173	YLR327C	225	YOL163W
18	YBR072W	70	YDR530C	122	YHL046C	174	YLR413W	226	YOL165C
19	YBR116C	71	YDR542W	123	YHR066W	175	YLR421C	227	YOR019W
20	YBR117C	72	YEL011W	124	YHR087W	176	YML004C	228	YOR031W
21	YBR126C	73	YEL039C	125	YHR138C	177	YML128C	229	YOR043W
22	YBR148W	74	YEL072W	126	YHR139C	178	YML131W	230	YOR095C
23	YBR199W	75	YER020W	127	YHR141C	179	YMR030W	231	YOR292C
24	YBR223C	76	YER042W	128	YHR146W	180	YMR090W	232	YOR298W
25	YBR296C	77	YER053C	129	YIL036W	181	YMR100W	233	YOR391C
26	YBR297W	78	YER056C	130	YIL045W	182	YMR105C	234	YOR394W

27	YBR298C	79	YER065C	131	YIL069C	183	YMR107W	235	YPL004C
28	YBR301W	80	YER066W	132	YIL077C	184	YMR139W	236	YPL014W
29	YCL051W	81	YER067W	133	YIL107C	185	YMR246W	237	YPL015C
30	YCR005C	82	YER078C	134	YIL136W	186	YMR255W	238	YPL043W
31	YCR072C	83	YER079W	135	YIL143C	187	YMR258C	239	YPL054W
32	YCR107W	84	YER117W	136	YIL153W	188	YMR262W	240	YPL093W
33	YDL022W	85	YER150W	137	YJL132W	189	YMR271C	241	YPL107W
34	YDL024C	86	YFL014W	138	YJL155C	190	YMR316W	242	YPL122C
35	YDL031W	87	YFL030W	139	YJL223C	191	YMR320W	243	YPL149W
36	YDL037C	88	YFL055W	140	YJR085C	192	YMR322C	244	YPL171C
37	YDL039C	89	YFL056C	141	YJR155W	193	YNL011C	245	YPL186C
38	YDL059C	90	YFL057C	142	YKL026C	194	YNL024C	246	YPL223C
39	YDL070W	91	YFR014C	143	YKL070W	195	YNL112W	247	YPL224C
40	YDL075W	92	YFR015C	144	YKL071W	196	YNL117W	248	YPL245W
41	YDL113C	93	YFR017C	145	YKL078W	197	YNL124W	249	YPL250C
42	YDL115C	94	YFR053C	146	YKL087C	198	YNL141W	250	YPL280W
43	YDL125C	95	YGL029W	147	YKL089W	199	YNL142W	251	YPL281C
44	YDL169C	96	YGL033W	148	YKL090W	200	YNL178W	252	YPL282C
45	YDL204W	97	YGL045W	149	YKL091C	201	YNL194C	253	YPR045C
46	YDL243C	98	YGL075C	150	YKL094W	202	YNL195C	254	YPR061C
47	YDR003W	99	YGL122C	151	YKL103C	203	YNL213C	255	YPR086W
48	YDR018C	100	YGL135W	152	YKL125W	204	YNL244C	256	YPR121W
49	YDR056C	101	YGL179C	153	YKL150W	205	YNL331C	257	YPR143W
50	YDR070C	102	YGL184C	154	YKL151C	206	YNR039C	258	YPR160W
51	YDR111C	103	YGL255W	155	YKL162C	207	YNR051C	259	YPR200C
52	YDR174W	104	YGL261C	156	YKL187C	208	YNR053C		

In addition, the present invention relates to a DNA fragment having a cold-inducible promoter function, which comprises

DNA described in the following (a) or (b):

(a) DNA existing in the non-translation region located upstream of the 5'-terminal side of a gene selected from the group consisting of genes of *Saccharomyces cerevisiae* described in Table 1, and comprising a deletion, substitution or addition of one or more nucleotides with respect to the DNA fragment having a cold-inducible promoter function; or

(b) DNA existing in the non-translation region located upstream of the 5'-terminal side of a gene selected from the group consisting of genes of *Saccharomyces cerevisiae* described in Table 1, and hybridizing with a DNA fragment consisting of a nucleotide sequence complementary to the DNA fragment having a cold-inducible promoter function under stringent conditions.

Moreover, the present invention relates to a DNA fragment, which comprises a cis sequence of the following (a) or (b), and has a cold-inducible promoter function:

(a) DNA sequence A: GCTCATCG; or

(b) DNA sequence B: GAGATGAG.

Furthermore, the present invention relates to a DNA fragment having a cold-inducible promoter function, which comprises DNA described in the following (a) or (b):

(a) DNA having the above cis sequence, and comprising a deletion, substitution or addition of one or more nucleotides with respect to the DNA fragment having a cold-inducible promoter function; or

(b) DNA having the above cis sequence, and hybridizing with a DNA fragment consisting of a nucleotide sequence complementary to the DNA fragment having a cold-inducible promoter function under stringent conditions.

Still further, the present invention relates to an expression vector comprising the above DNA fragment, or an expression vector characterized in that it comprises a foreign gene or foreign DNA fragment downstream of the above DNA fragment in the above expression vector.

Still further, the present invention relates to a transformant transformed with the above expression vector. An example of a host is yeast.

Still further, the present invention relates to a method for producing a protein or a method for regulating RNA production, which is characterized in that it comprises decreasing a culture temperature and culturing the transformant at the decreased temperature. An example of a culture temperature is 10°C or lower.

The present invention will be described in detail below. The present application claims priority from Japanese Patent Application No. 2002-191383 filed on June 28, 2002. This specification includes part or all of the contents as disclosed in the specification and/or drawings of the above Japanese Patent Application.

By identifying a cold-inducible gene of yeast, the DNA fragment of the present invention having a cold-inducible promoter function of yeast can be identified. Genes, the amount of mRNA of which is increased when the culture temperature is decreased from 30°C,

an optimal culture temperature for yeast, to 10°C, are identified as cold-inducible genes. In order to completely capture these cold-inducible genes, approximately 5,800 genes are obtained by eliminating genes, whose preparation is difficult for reasons such as amplification or the like, from all genes (approximately 6,200) derived from *Saccharomyces cerevisiae*. Thereafter, cDNA derived from each of the 5,800 genes is fixed on a slide glass, so as to prepare a DNA microarray (manufactured by DNA Chip Research Inc.). As RNA samples allowing to act on the DNA microarray, multiple RNA samples prepared by recovering a cell mass over time after decreasing the culture temperature of *Saccharomyces cerevisiae* from 30°C to 10°C and then extracting RNA from the recovered cell mass can be used. Using the thus prepared multiple samples, genes whose expression level increases immediately after shifting the culture temperature of *Saccharomyces cerevisiae* to a low temperature, and genes whose expression level gradually increases, can be identified. Using these RNA samples, the mRNA amount of each gene fixed on a DNA microarray is compared between before and after a low temperature treatment, so that a gene whose mRNA amount after the low temperature treatment is greater than the mRNA amount before the low temperature treatment can be identified as a cold-inducible gene. For example, a gene whose mRNA amount after a low temperature treatment is 3 times or more greater than the mRNA amount before the low temperature treatment can be identified as a cold-inducible gene. The thus identified 259 genes which are novel as a cold-inducible gene are shown in the following Table 2.

Table 2

No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name	No.	Systematic gene name
1	YAL014C	53	YDR184C	105	YGR008C	157	YKL224C	209	YNR071C
2	YAL015C	54	YDR219C	106	YGR043C	158	YKR049C	210	YNR075W
3	YAL025C	55	YDR253C	107	YGR053C	159	YKR075C	211	YNR076W
4	YAL034C	56	YDR256C	108	YGR088W	160	YKR077W	212	YOL002C
5	YBL048W	57	YDR262W	109	YGR102C	161	YKR100C	213	YOL016C
6	YBL049W	58	YDR306C	110	YGR154C	162	YLL055W	214	YOL084W
7	YBL054W	59	YDR336W	111	YGR197C	163	YLL056C	215	YOL101C
8	YBL056W	60	YDR346C	112	YGR222W	164	YLR009W	216	YOL108C
9	YBL065W	61	YDR387C	113	YGR223C	165	YLR145W	217	YOL116W
10	YBL078C	62	YDR398W	114	YGR251W	166	YLR149C	218	YOL124C
11	YBR016W	63	YDR435C	115	YGR256W	167	YLR164W	219	YOL127W
12	YBR018C	64	YDR453C	116	YGR262C	168	YLR251W	220	YOL132W

13	YBR024W	65	YDR471W	117	YGR286C	169	YLR252W	221	YOL153C
14	YBR034C	66	YDR492W	118	YGR294W	170	YLR266C	222	YOL154W
15	YBR045C	67	YDR496C	119	YHL016C	171	YLR311C	223	YOL161C
16	YBR047W	68	YDR504C	120	YHL021C	172	YLR312C	224	YOL162W
17	YBR050C	69	YDR516C	121	YHL036W	173	YLR327C	225	YOL163W
18	YBR072W	70	YDR530C	122	YHL046C	174	YLR413W	226	YOL165C
19	YBR116C	71	YDR542W	123	YHR066W	175	YLR421C	227	YOR019W
20	YBR117C	72	YEL011W	124	YHR087W	176	YML004C	228	YOR031W
21	YBR126C	73	YEL039C	125	YHR138C	177	YML128C	229	YOR043W
22	YBR148W	74	YEL072W	126	YHR139C	178	YML131W	230	YOR095C
23	YBR199W	75	YER020W	127	YHR141C	179	YMR030W	231	YOR292C
24	YBR223C	76	YER042W	128	YHR146W	180	YMR090W	232	YOR298W
25	YBR296C	77	YER053C	129	YIL036W	181	YMR100W	233	YOR391C
26	YBR297W	78	YER056C	130	YIL045W	182	YMR105C	234	YOR394W
27	YBR298C	79	YER065C	131	YIL069C	183	YMR107W	235	YPL004C
28	YBR301W	80	YER066W	132	YIL077C	184	YMR139W	236	YPL014W
29	YCL051W	81	YER067W	133	YIL107C	185	YMR246W	237	YPL015C
30	YCR005C	82	YER078C	134	YIL136W	186	YMR255W	238	YPL043W
31	YCR072C	83	YER079W	135	YIL143C	187	YMR258C	239	YPL054W
32	YCR107W	84	YER117W	136	YIL153W	188	YMR262W	240	YPL093W
33	YDL022W	85	YER150W	137	YJL132W	189	YMR271C	241	YPL107W
34	YDL024C	86	YFL014W	138	YJL155C	190	YMR316W	242	YPL122C
35	YDL031W	87	YFL030W	139	YJL223C	191	YMR320W	243	YPL149W
36	YDL037C	88	YFL055W	140	YJR085C	192	YMR322C	244	YPL171C
37	YDL039C	89	YFL056C	141	YJR155W	193	YNL011C	245	YPL186C
38	YDL059C	90	YFL057C	142	YKL026C	194	YNL024C	246	YPL223C
39	YDL070W	91	YFR014C	143	YKL070W	195	YNL112W	247	YPL224C
40	YDL075W	92	YFR015C	144	YKL071W	196	YNL117W	248	YPL245W
41	YDL113C	93	YFR017C	145	YKL078W	197	YNL124W	249	YPL250C
42	YDL115C	94	YFR053C	146	YKL087C	198	YNL141W	250	YPL280W
43	YDL125C	95	YGL029W	147	YKL089W	199	YNL142W	251	YPL281C
44	YDL169C	96	YGL033W	148	YKL090W	200	YNL178W	252	YPL282C
45	YDL204W	97	YGL045W	149	YKL091C	201	YNL194C	253	YPR045C
46	YDL243C	98	YGL075C	150	YKL094W	202	YNL195C	254	YPR061C
47	YDR003W	99	YGL122C	151	YKL103C	203	YNL213C	255	YPR086W
48	YDR018C	100	YGL135W	152	YKL125W	204	YNL244C	256	YPR121W
49	YDR056C	101	YGL179C	153	YKL150W	205	YNL331C	257	YPR143W
50	YDR070C	102	YGL184C	154	YKL151C	206	YNR039C	258	YPR160W
51	YDR111C	103	YGL255W	155	YKL162C	207	YNR051C	259	YPR200C
52	YDR174W	104	YGL261C	156	YKL187C	208	YNR053C		

The DNA fragment of the present invention exists in the non-translation region located upstream of the 5'-terminal side of a gene selected from the group consisting of genes of *Saccharomyces cerevisiae* described in the above Table 2, and functions as a cold-inducible promoter.

Table 2 shows numbers from 1 to 259 imparted to 259 genes in association with systematic gene names thereof. These systematic gene names correspond to the names registered as systematic names in yeast genome database (*Saccharomyces cerevisiae* genome database; http://genome-www.stanford.edu/Saccharomyces/). Accordingly, the genes of *Saccharomyces cerevisiae* described in the above Table 2 can easily be specified by using such a systematic gene name as a key and searching for the systematic name through the yeast genome database. Moreover, the nucleotide sequences of the genes of *Saccharomyces cerevisiae* described in Table 2 can be obtained by searching through the yeast genome database. Furthermore, other types of information regarding the genes of *Saccharomyces cerevisiae* described in Table 2 can also be obtained by searching through the yeast genome database.

The term "a cold-inducible promoter" means a promoter exhibiting higher promoter activity at a temperature lower than the optimal culture temperature for yeast as compared to the promoter activity obtained at the optimal culture temperature for yeast. More specifically, such a cold-inducible promoter exhibits 3 times or more higher promoter activity at a temperature lower than the optimal culture temperature for yeast as compared to the promoter activity obtained at the optimal culture temperature for yeast. Herein, the optimal culture temperature for yeast is approximately 30°C. In addition, the term "a temperature lower than the optimal culture temperature for yeast" means a temperature lower than 30°C, and for example, approximately 10°C. However, if the above temperature is a temperature of 20°C or lower, and preferably 15°C or lower, it is not limited to approximately 10°C..

Promoter activity can be measured according to conventional methods. For example, an expression vector, in which a reporter gene is ligated downstream of a promoter such that the gene can be expressed, is constructed. Subsequently, a suitable host (e.g. yeast) is transformed with the expression vector. The obtained transformant is cultured under certain conditions, and the expression level of the reporter gene can be assayed at a level of mRNA or protein, so as to measure promoter activity under the above-described conditions.

The term "non-translation region located upstream of the 5'-terminal side of a gene" means a region, which exists on the 5'-terminal side of the coding strand of a gene specified as

stated above and is not translated into a protein. In other words, such a non-translation region means a region that is not included in what is called ORF (open reading frame).

The non-translation region located upstream of 5'-terminal side of a certain gene (hereinafter referred to as a target gene) can specifically be identified using the yeast genome database. That is to say, first, a search is performed through the yeast genome database using the systematic gene name of a target gene as a key. As a result of the search, various types of information regarding the target gene are obtained. Using various types of information, the position of the target gene on a chromosome is determined. Thereafter, on the basis of the position of the target gene on a chromosome, a gene located upstream of the 5'-terminal side of the target gene (referred to as a 5' upstream adjacent gene) is specified from the chromosome map registered in the yeast genome database. A region sandwiched between the thus specified target gene and 5' upstream adjacent gene is a region that is neither translated into a protein, nor contains ORF. Thus, the region sandwiched between the target gene and 5' upstream adjacent gene can be specified by the above-described processes as a non-translation region on the 5'-terminal side of the target gene.

The nucleotide sequence of the thus specified non-translation region on the 5'-terminal side of the target gene can be obtained by searching information regarding total nucleotide sequences of yeast genome registered in the yeast genome database. In addition, the specified non-translation region on the 5'-terminal side of the target gene can easily be obtained by performing PCR using the genome extracted form yeast as a template and also using primers complementary to the nucleotide sequences at both termini of the above region consisting of approximately 20 nucleotides.

The DNA fragment of the present invention may be either the entire non-translation region on the 5'-terminal side, or a portion of the non-translation region on 5'-terminal side as long as it has a function as a cold-inducible promoter.

Moreover, the DNA fragment of the present invention may be a DNA fragment, which comprises DNA comprising a deletion, substitution or addition of one or several (for example, 1 to 10, or 1 to 5) nucleotides with respect to the above DNA fragment and has a cold-inducible promoter function.

Furthermore, in the DNA fragment of the present invention included is a DNA fragment, which hybridizes with a DNA fragment consisting of a nucleotide sequence complementary to the above DNA fragment under stringent conditions and has a cold-inducible promoter function.

Herein, when probe DNA labeled with phosphorus-32 is used, the term "stringent conditions" is used to mean hybridization performed in a hybridization solution consisting of 5 x SSC (0.75 M NaCl, 0.75 M sodium citrate), 5 x Denhardt's reagent (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), and 0.1% sodium dodecyl sulfate (SDS), at a temperature between 45°C and 65°C, and preferably between 55°C and 65°C. In addition, in a washing step, washing is performed in a washing solution consisting of 2 x SSC and 0.1% SDS at a temperature between 45°C and 55°C, and more preferably, washing is performed in a washing solution consisting of 0.1 x SSC and 0.1% SDS at a temperature between 45°C and 55°C. When probe DNA labeled with an enzyme using an AlkPhos direct labeling module kit (Amersham Biotech) is used, hybridization is carried out in a hybridization solution (containing 0.5 M NaCl and a 4% blocking reagent), the composition of which is described in a manual attached with the kit, at a temperature between 55°C and 75°C. In addition, in a washing step, washing is performed in a first washing solution (containing 2 M urea) described in the manual attached with the kit at a temperature between 55°C and 75°C, and then in a second washing solution at room temperature. Furthermore, other detection methods may also be applied. When other detection methods are applied, standard conditions for the applied detection method may be used.

The DNA fragment of the present invention may be a DNA fragment, which comprises DNA described in the following (a) or (b) and has a cold-inducible promoter function:

(a) DNA sequence A: GCTCATCG; or

(b) DNA sequence B: GAGATGAG.

The DNAs described in the above (a) and (b) are sequences (referred to as cis sequences) that are common in the non-translation regions located upstream of the 5'-terminal sides of genes exhibiting cold inducibility at an early stage, which are identified by the above-described method using the above-described DNA microarray. For example,

regarding genes exhibiting cold inducibility at an early stage, the culture temperature is first decreased to 10°C. Then, 15 minutes later, genes whose signal is 2 times or more increased can be identified as genes exhibiting cold inducibility at an early stage. The identified 41 genes are shown in the following Table 3.

Table 3

No.	Systematic gene name	No.	Systematic gene name
1	YDL039C	22	YDL063C
2	YNL141W	23	YOR360C
3	YDL037C	24	YHR196W
4	YKR075C	25	YNL065W
5	YER056C	26	YHR066W
6	YOL124C	27	YLR407W
7	YDR492W	28	YOR101W
8	YLR413W	29	YNL112W
9	YCR072C	30	YGR159C
10	YOR095C	31	YGL055W
11	YNL175C	32	YNR053C
12	YDR398W	33	YPL093W
13	YGR283C	34	YHR170W
14	YBR296C	35	YHR148W
15	YDR184C	36	YBR034C
16	YOR338W	37	YOL010W
17	YAL025C	38	YKL078W
18	YOR063W	39	YMR290C
19	YIL096C	40	YDR101C
20	YER127W	41	YBL054W
21	YBL042C		

Table 3 shows numbers from 1 to 41 imparted to 41 genes in association with systematic gene names thereof. As in the case of Table 2, these systematic gene names correspond to the names registered as systematic names in the above-described yeast genome database.

Subsequently, using Gene Spring (Silicon Genetics), cis sequences existing between the ORF and 600 bp upstream of individual genes are searched. As a result, common DNA sequences existing in some of these genes can be obtained. Specifically, the above DNA

sequence A is a common cis sequence that can be found in YNL112W, YGR159C, YGL055W, YNR053C, YPL093W, YHR170W, and YHR148W (which correspond to Nos. 29 to 35 in Table 3), and the above DNA sequence B is a common cis sequence that can be found in YBR034C, YOL010W, YKL078W, YMR290C, YDR101C, and YBL054W (which correspond to Nos. 36 to 41 in Table 3).

Further, the above DNA fragment may be a DNA fragment, which comprises DNA comprising a deletion, substitution or addition of one or several nucleotides (for example, 1 to 3) with respect to the above DNA fragment, and has a cold-inducible promoter function.

Furthermore, a DNA fragment comprising DNA hybridizing with a DNA fragment consisting of a nucleotide sequence complementary to the above DNA fragment under stringent conditions and having a cold-inducible promoter function may also be included in the DNA fragment of the present invention.

Herein, when probe DNA labeled with phosphorus-32 is used, the term "stringent conditions" is used to mean hybridization performed in a hybridization solution consisting of 5 x SSC (0.75 M NaCl, 0.75 M sodium citrate), 5 x Denhardt's reagent (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), and 0.1% sodium dodecyl sulfate (SDS), at a temperature between 45°C and 65°C, and preferably between 55°C and 65°C. In addition, in a washing step, washing is performed in a washing solution consisting of 2 x SSC and 0.1% SDS at a temperature between 45°C and 55°C, and more preferably, washing is performed in a washing solution consisting of 0.1 x SSC and 0.1% SDS at a temperature between 45°C and 55°C. When probe DNA labeled with an enzyme using an AlkPhos direct labeling module kit (Amersham Biotech) is used, hybridization is carried out in a hybridization solution (containing 0.5 M NaCl and a 4% blocking reagent), the composition of which is described in a manual attached with the kit, at a temperature between 55°C and 75°C. In addition, in a washing step, washing is performed in a first washing solution (containing 2 M urea) described in a manual attached with the kit at a temperature between 55°C and 75°C, and then in a second washing solution at room temperature. Furthermore, other detection methods may also be applied. When other detection methods are applied, standard conditions for the applied detection method may be used.

Once the nucleotide sequence of the DNA fragment of the present invention is established, then the DNA fragment of the present invention can be obtained by chemical synthesis, by performing PCR using the cloned probe as a template, or by hybridization of a DNA fragment having the above nucleotide sequence as a probe. Moreover, even in the case of a mutant of the DNA fragment of the present invention, a site-directed mutagenesis or other techniques can be applied, so as to synthesize a fragment having the same functions as those of a DNA fragment before mutation.

In order to introduce mutation into the DNA fragment of the present invention, known methods such as Kunkel method or Gapped duplex method, or methods equivalent thereto, can be applied. For example, mutation can be introduced by using a kit for introducing mutation (e.g. Mutant-K (manufactured by Takara) or Mutant G (manufactured by Takara)) using the site-directed mutagenesis, or by using a series of LA PCR *in vitro* Mutagenesis kits manufactured by Takara.

The expression vector of the present invention can be obtained by inserting the DNA fragment of the present invention into a suitable vector. A vector into which the DNA fragment of the present invention is inserted is not particularly limited, as long as it can replicate itself in a host. Examples of such a vector may include a plasmid, a shuttle vector, and a helper plasmid. When a vector has no self-replicating ability, a DNA fragment, which can replicate itself when it is inserted into the chromosome of a host, may be used.

Examples of plasmid DNA may include plasmids derived from *Escherichia coli* (e.g. pBR322, pBR325, pUC118, pUC119, pUC18, pUC19, and pBluescript), plasmids derived from *Bacillus subtilis* (e.g. pUB110 and pTP5), and plasmids derived from yeast (e.g. YEp system such as YEp13, and YCp system such as YCp50). Examples of phage DNA may include λ phages (e.g. Charon 4A, Charon 21A, EMBL 3, EMBL 4, λ gt10, λ gt11, and λ ZAP). Moreover, animal viruses such as retrovirus or vaccinia virus, and insect viruses such as baculovirus, may also be used as viral vectors.

In order to insert the DNA fragment of the present invention into a vector, a method comprising, first cleaving the purified DNA with suitable restriction enzymes, and then inserting the obtained DNA portion into a restriction site or multicloning site of suitable vector

DNA, and ligating it to the vector, is applied. Otherwise, it may also be possible that both vector and the DNA fragment of the present invention be allowed to have a portion of homologous regions, and that both be ligated by the *in vitro* method using PCR and the like, or by the *in vivo* method using yeast and the like.

The expression vector of the present invention may further comprise a foreign gene or foreign DNA fragment, which is inserted downstream of the DNA fragment of the present invention. A method of inserting such a foreign gene or foreign DNA fragment into a vector is the same as the method of inserting the DNA fragment of the present invention into a vector.

Any protein or peptide may be used as such a foreign gene located downstream of the DNA fragment of the present invention in the expression vector of the present invention. An example may be a protein that is particularly suitable for production at a low temperature. More specifically, examples of such a protein may include an antifreeze protein functioning at a low temperature, a cold-active enzyme that is thermolabile and is likely to denature due to heat, and a fluorescent protein GFP. Furthermore, examples of a foreign DNA fragment located downstream of the DNA fragment of the present invention may include antisense RNA and ribozyme, wherein RNA functions by itself.

The transformant of the present invention can be obtained by introducing the expression vector of the present invention into a host. A host is not particularly limited herein, as long as it can allow a promoter and a foreign gene to express. In the present invention, an example of the host may be yeast. Examples of such yeast may include *Saccharomyces cerevisiae*, experimental yeast, brewer's yeast, edible yeast, and industrial yeast.

A method of introducing the expression vector of the present invention into yeast is not particularly limited, as long as it is a method of introducing DNA into yeast. Examples of such a method may include electroporation, the spheroplast method, and the lithium acetate method. In addition, it may also be a yeast transformation method, which involves substitution or insertion into a chromosome, using a vector such as YIp system or a DNA sequence homologous to a certain region in a chromosome. Furthermore, any methods

described in common experimental manuals or scientific papers may be applied as methods of introducing the expression vector of the present invention into a yeast cell.

The expression vector of the present invention is not only introduced into the aforementioned yeast hosts, but it can be also introduced into bacteria belonging to the genus Escherichia such as *Escherichia coli*, the genus Bacillus such as *Bacillus subtilis*, or the genus Pseudomonas such as *Pseudomonas putida*, animal cells such as COS cells, insect cells such as Sf9, or plants belonging to Brassicaceae, so as to obtain a transformant. When a bacterium is used as a host, it is preferable that the expression vector of the present invention be able to self-replicate in the bacterium, and also that it be composed of the DNA fragment of the present invention, a ribosome-binding sequence, a gene of interest, and a transcription termination sequence. In addition, a gene regulating a promoter may also be comprised in the expression vector.

A method of introducing the expression vector of the present invention into a bacterium is not particularly limited, as long as it is a method of introducing DNA into a bacterium. Examples of such a method may include a method of using calcium ions and electroporation.

When an animal cell is used as a host, a monkey cell COS-7, Vero, a Chinese hamster ovary cell (CHO cell), a mouse L cell, or the like is used. Examples of a method of introducing the expression vector of the present invention into an animal cell may include electroporation, the calcium phosphate method, and lipofection.

When an insect cell is used as a host, an Sf9 cell or the like is used. Examples of a method of introducing the expression vector of the present invention into an insect cell may include the calcium phosphate method, lipofection, and electroporation.

When a plant is used as a host, a plant body as a whole, a plant organ (e.g. a leaf, a petal, a stem, a root, and a seed), a plant tissue (e.g. epidermis, phloem, parenchyma, xylem, and vascular bundle), a plant cultured cell, or the like is used. Examples of a method of introducing the expression vector of the present invention into a plant may include electroporation, the Agrobacterium method, particle gun, and the PEG method.

Incorporation of a gene into a host can be confirmed by PCR, Southern hybridization, Northern hybridization, and other methods. For example, DNA is prepared from a

transformant, DNA-specific primers are designed, and PCR is then carried out. Thereafter, the amplified product is subjected to agarose gel electrophoresis, polyacrylamide gel electrophoresis, capillary electrophoresis, etc., followed by staining with ethidium bromide, SYBR Green solution, or the like. Thereafter, the amplified product is detected as a single band, so as to confirm that transformation has been carried out. Also, PCR can be carried out using primers that have previously been labeled with fluorescent dye or the like, so as to detect an amplified product. Further, a method of binding an amplified product to a solid phase such as a microplate, and then confirming the amplified product by a fluorescent or enzyme reaction may also be adopted.

The method of the present invention for producing a protein comprises: introducing into a host an expression vector comprising the DNA fragment of the present invention and a foreign gene ligated downstream of the above DNA fragment, so as to prepare a transformant; and decreasing a culture temperature and culturing the transformant at the decreased temperature, so as to produce a protein encoded by the foreign gene located downstream thereof. An example of such a culture temperature may be 10°C or lower. Since, for example, among cold-active enzymes or antifreeze proteins which some organisms living in a low temperature area have, these cold-active enzymes or antifreeze proteins can be extremely thermolabile, they may be denatured when they are produced at an ordinary temperature. In such a case, an expression vector, which comprises a gene encoding the aforementioned cold-active enzyme or antifreeze protein that is ligated downstream of the cold-inducible promoter of the present invention, is introduced into yeast, and the temperature in this system is decreased from approximately 30°C as an optimal culture temperature for yeast to a lower temperature (for example, 10°C), so that the amount of mRNA corresponding to the gene ligated downstream of the DNA fragment of the present invention can be increased and that an expression system for efficiently expressing an active protein can be constructed.

When a protein (enzyme) to be produced causes cell damage, such as the case of protease, since it inhibits the growth of a recombinant, it is extremely difficult to produce such a protein (enzyme). In this case, according to the protein production method of the present invention, a recombinant is first allowed to grow, while the production amount of a foreign

gene product is limited at an optimal culture temperature (approximately 30°C). Thereafter, at the time when a sufficient amount of cell mass is obtained, the temperature can be decreased, thereby inducibly producing a foreign gene product while suppressing cytotoxicity. Moreover, with regard to a fluorescent protein GFP that has frequently been used for kinetic analysis of intracellular proteins or biomonitoring in recent years, it has been known that when the protein is produced in a recombinant, it requires a maturation process of changing its structure into a protein structure for emitting fluorescence. It is considered that this maturation process is promoted at a low temperature. As a matter of fact, when the protein is produced at a temperature lower than the ordinary culture temperature, a higher amount of fluorescence can be obtained (Matsuzaki et al., a supplementary volume of *Jikken Igaku, post genome jidai no jikken koza 3, "GFP to bioimaging,"* Yodosha Co., Ltd., (2000) pp. 31-37). Thus, the protein production method of the present invention enables biomonitoring whereby GFP is used at higher sensitivity.

Moreover, the method of the present invention for regulating RNA production comprises: preparing an expression vector comprising the DNA fragment of the present invention and a foreign DNA fragment ligated downstream of the above DNA fragment; introducing the expression vector into a host, so as to prepare a transformant; and decreasing a culture temperature and culturing the transformant at the decreased temperature, so that RNA production can be regulated by the foreign DNA fragment located downstream thereof. An example of such a culture temperature may be 10°C or lower. For example, an expression vector, which comprises the cold-inducible promoter of the present invention and a gene encoding antisense RNA to a specific gene ligated downstream of the above promoter, is introduced into yeast, and the temperature in this system is decreased from approximately 30°C as an optimal culture temperature for yeast to a lower temperature (for example, 10°C), so that the amount of antisense RNA corresponding to the gene ligated downstream of the DNA fragment of the present invention can be increased and that the expression of the specific gene can be regulated.

Brief Description of the Drawings

Figure 1 shows the results of Northern blotting analysis showing a change in the amount of HSP12 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 2 shows the results of Northern blotting analysis showing a change in the amount of DBP2 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 3 shows the results of Northern blotting analysis showing a change in the amount of NSR1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 4 shows the results of Northern blotting analysis showing a change in the amount of AAH1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 5 shows the results of Northern blotting analysis showing a change in the amount of YKR075C mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 6 shows the results of Northern blotting analysis showing a change in the amount of OLE1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 7 shows the results of Northern blotting analysis showing a change in the amount of ACT1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 8 shows the structure of a plasmid obtained by ligating a DNA fragment having a DBP2 promoter function upstream of the 5'-terminal side of EGFP DNA, using pUG35-MET25 as a reporter vector. The position of a DNA sequence A (GCTCATCG) and the positions of Inverse PCR primers for removing the above DNA sequence (wherein RPC19-DBP2 IGR-cis F and RPC19-DBP2 IGR-cis R are abbreviated as DBP2-cis F and DBP2-cis R, respectively) are also shown in the figure;

Figure 9 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having a DBP2 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 10 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having a DBP2 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 11 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HMT1 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 12 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HMT1 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 13 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HSP12 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C.

The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 14 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HSP12 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 15 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA, which is obtained, when a DNA fragment having a modified DBP2 promoter function (right) obtained by removing a DNA sequence A (GCTCATCG) from a DNA fragment having a DBP2 promoter function comprising the above DNA sequence A and a native DNA fragment having a DBP2 promoter function (left) are used, and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 16 shows the results of Northern blotting analysis showing a change in the amount of EGFP mRNA, which is obtained, when a DNA fragment having a modified HMT1 promoter function (right) obtained by removing a DNA sequence B (GAGATGAG) from a DNA fragment having an HMT1 promoter function comprising the above DNA sequence B and a native DNA fragment having a HMT1 promoter function (left) are used, and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane;

Figure 17 shows, in the upper case, a plasmid construct comprising an ADH1 promoter, a TDH3 promoter, or a DNA fragment having an HSP12 cold-inducible promoter function, and in the middle and lower cases, the results of Northern blotting analysis, which is performed to compare the transcriptional activity of a DNA fragment having an HSP12 cold-inducible promoter function with the transcriptional activities of an ADH1 promoter and

a TDH3 promoter in yeast. The amount of EGFP mRNA is represented by the density and size of dots in each lane;

Figure 18 shows, in the upper case, a plasmid construct comprising a TDH3 promoter or a DNA fragment having an HSP12 cold-inducible promoter function, and in the lower case, the results of Western blotting analysis, which is performed to compare the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function with the protein-producing ability of a TDH3 promoter in yeast. The amount of an EGFP protein is represented by the density and size of dots in each lane;

Figure 19 shows, in the upper case, an expression plasmid construct, which is obtained by inserting an expression cassette comprising a DNA fragment having an HSP12 cold-inducible promoter function, the ORF of EGFP, and a CYC1 terminator, into pUG35 having a centromere as a replication origin, pYES2 having 2 μ as a replication origin, or pYEX-BX having 2 μ as a replication origin and having a weak leucine synthetase gene (leu2-d), from each of which an original promoter has been removed, and in the middle and lower cases, the results of Northern blotting analysis showing the fact that the ability of the transcriptional activation of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the structure of a plasmid in itself. The amount of EGFP mRNA is represented by the density and size of dots in each lane;

Figure 20 shows the results of SDS-PAGE analysis showing the fact that the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the structure of a plasmid in itself. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane;

Figure 21 shows the results of SDS-PAGE analysis showing the fact that the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the type of yeast strain *Saccharomyces cerevisiae*. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane;

Figure 22 shows the results of SDS-PAGE analysis showing the fact that the protein-producing ability of an expression vector comprising a DNA fragment having an

HSP12 cold-inducible promoter function is more excellent than that of the existing expression vector of yeast. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane;

Figure 23 shows the results of SDS-PAGE analysis showing the fact that the protein-producing ability of an expression vector comprising a DNA fragment having an HSP12 cold-inducible promoter function is induced in a wide low temperature range. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane;

Figure 24 shows the results of Western blotting analysis showing the fact that a cassette comprising an HSP12 promoter, the ORF of EGFP, and a CYC1 terminator was incorporated into methylotrophic yeast, *Pichia pastoris*, so that an EGRP protein was inducibly produced in *Pichia pastoris* at a low temperature;

Figure 25 shows the results of Western blotting analysis showing the fact that an antifreeze protein RD3 is expressed as a soluble protein by a DNA fragment having an HSP12 cold-inducible promoter function. The amount of an RD3 protein is represented by the density and size of a band in each lane; and

Figure 26 shows results obtained by expressing two types of fluorescent proteins, ECFP and DsRed by cold induction using pLTex321.

Best Mode for Carrying Out the Invention

[Examples]

The present invention will be further specifically described in the following examples. However, the examples are not intended to limit the technical scope of the invention.

Example 1 Identification of cold-inducible gene

A yeast strain, *Saccharomyces cerevisiae* YPH500 (purchased from Stratagene) was inoculated into 10 ml of YEPD medium (2% bactopeptone, 1% bactoyeast extract, 2% glucose), using an inoculating loop, followed by a shake culture at 30°C for 2 days. 5 ml of the obtained culture solution was then inoculated into 1,000 ml of YEPD medium, followed by

a shake culture at 30°C, until the absorbance at 600 nm became approximately 2 (Culture solution 1).

Fifty ml of a solution was separated from Culture solution 1, and cells were collected (a pre-low temperature treatment sample). The yeast cell mass was then frozen with liquid nitrogen, and the frozen cell mass was conserved at -80°C in a deep freezer, until the time when RNA was prepared. The residue of Culture solution 1 was rapidly immersed in a shake water bath, which had previously been set at 10°C, and it was shaken for 30 minutes for quenching. Subsequently, the resultant product was transferred to a low temperature thermostat, which had previously been set at 10°C, and a shake culture was continuously carried out at 10°C. The time when the culture solution was immersed in a shake water bath at 10°C was determined at 0 minute, and 50 ml each of the culture solution (a post-low temperature treatment sample) was separated by the same method as described above, 15 minutes, 30 minutes, 2 hours, 4 hours, and 8 hours later. Every time, a yeast cell mass was recovered and conserved at -80°C.

Preparation of RNA from the recovered yeast cell mass was carried out by the hot phenol method. Ten ml of an NaOAc/SDS solution (20 mM NaOAc (pH 5.5), 0.5% SDS, 1 mM EDTA), which had previously been heated to 65°C, was added to the recovered yeast cell mass. Thereafter, 20 mM NaOAc (pH 5.5)-saturated phenol, which had been heated to 65°C, was further added thereto. The mixture was fully stirred at 65°C for 10 minutes, and it was then cooled on ice for 5 minutes. The mixture was centrifuged to recover a water phase, and 30 ml of ethanol was then added thereto, followed by cooling at -80°C for 30 minutes. The resultant product was centrifuged to recover RNA. After a supernatant was discarded, 70% ethanol was added to the residue to wash it. The resultant product was centrifuged again, so that RNA was recovered as a precipitate. The obtained RNA was dissolved in 1 ml of NaOAc/SDS, followed by performing phenol extraction twice. Subsequently, 500 µl of 2-propanol was added thereto, and the mixture was then cooled at -80°C for 20 minutes. Thereafter, the mixture was centrifuged to recover RNA. The residue was washed with 70% ethanol, as described above. RNA recovered as a precipitate was dissolved in 200 µl of NaOAc/SDS, and ethanol precipitation and washing with 70% ethanol were carried out, as

described above. Finally, RNA was dissolved in 200 μl of distilled water. Qiagen RNeasy Mini Kit (Qiagen) was used to eliminate small molecule RNA, and RNA was purified in accordance with the protocol attached with the kit.

DNA labeled with fluorescent dye was produced using 15 μg of the thus prepared yeast total RNA and 5 μg of oligo (dT) in accordance with the manual prepared by DNA Chip Research Inc. As fluorescent dye markers, Cy3-dUTP (a pre-low temperature treatment sample) and Cy5-dUTP (a post-low temperature treatment sample), which were manufactured by Amersham Biotech, were used. Hybridization of a DNA microarray with a labeled cDNA was carried out in accordance with the manual prepared by DNA Chip Research Inc.

Hybridization was carried out. The washed DNA microarray was analyzed using GenePix4000A and Gene Pix Pro programs manufactured by Axon. The intensities of fluorescences derived from Cy3 and Cy5, which hybridized with each gene spotted on the DNA microarray, were measured. Thereafter, the obtained data was analyzed using a Gene Spring program manufactured by Silicon Genetics, so as to carry out the equalization, standardization, and time series analysis of the data. The operations were carried out in accordance with a manual attached with the program.

As a result of the analysis, a gene spot, regarding which the fluorescence intensity of Cy5 (a post-low temperature treatment sample) was 3 times or more higher than that of Cy3 (a pre-low temperature treatment sample) at any time of 15 minutes, 30 minutes, 2 hours, 4 hours, and 8 hours, was determined to be a gene controlled by a cold-inducible promoter. Using an arrangement plan of genes provided from DNA Chip Research Inc., the name of the gene was specified. The thus identified genes which are novel as a cold-inducible gene are shown in the following Table 4.

Table 4

Genes exhibiting 3 times or more of cold inducibility

No.	Systematic gene name	Common name	0.25 hr (Normalized)	0.5 hr (Normalized)	2 hr (Normalized)	4 hr (Normalized)	8 hr (Normalized)
1	YAL014C		1.0033	1.38948	1.72818	2.9439597	4.03293
2	YAL015C	NTG1	1.30889	1.37036	2.00195	2.6074	3.3652697
3	YAL025C	MAK16	2.23906	3.4162998	2.42264	1.12295	0.8204
4	YAL034C	FUN19	0.6976	0.74361	2.20462	4.08124	1.68092
5	YBL048W		0.88448	0.8266	1.09091	2.02884	3.3299603
6	YBL049W		1.03575	1.05359	1.34462	2.65459	4.5075
7	YBL054W		2.15287	3.11488	1.74043	0.62387	0.52929
8	YBL056W	PTC3	1.1519	1.17583	2.13383	2.88587	4.9089103
9	YBL065W		0.86021	0.96902	1.54386	2.37517	4.7262497
10	YBL078C	AUT7	1.18513	1.2101	1.48343	2.94463	5.8092504
11	YBR016W		0.98765	0.9521	1.62529	3.0799499	2.9415097
12	YBR018C	GAL7	0.66284	0.62314	0.7807	1.93283	3.0417998
13	YBR024W	SCO2	1.24344	1.20799	1.80866	3.0938697	3.6857402
14	YBR034C	HMT1	2.63514	3.09186	0.81714		0.6994
15	YBR045C	GIP1	0.95867	1.00611	2.66207	5.17561	3.44039
16	YBR047W		1.05117	0.92493	1.34783	2.19222	3.0629797
17	YBR050C	REG2	1.54439	2.35019	4.04587	6.1531	1.82178
18	YBR072W	HSP26	1.14509	1.03625	0.67711	1.38069	3.6218097
19	YBR116C		0.8781	0.90162	0.87838	5.7050705	7.27097
20	YBR117C	TKL2	0.86577	0.81363	2	3.31154	7.0771995
21	YBR126C	TPS1	0.91332	0.74795	1.15979	2.61072	3.6894302
22	YBR148W	YSW1	0.89222	1.07221	1.66102	3.1233997	1.70299
23	YBR199W	KTR4	1.0203	0.98227	1.44268	1.95379	3.15104
24	YBR223C		0.88953	0.87627	1.36441	3.0317502	3.5880897
25	YBR296C	PHO89	2.39344	5.2771997	8.24286	6.0016	1.35429
26	YBR297W	MAL33	1.6135	2.16031	1.89716	2.53522	4.09979
27	YBR298C	MAL31	1.03296	1.30158	1.65693	2.67207	3.10517
28	YBR301W		0.87094	0.98242	1.74468	3.0934799	2.27173
29	YCL051W	LRE1	0.77913	0.98816	2.06154	3.2779498	3.2061903
30	YCR005C	CIT2	0.84784	0.8432	1.74417	2.0718	3.03626
31	YCR072C		2.54439	3.15586	1.28448	0.40795	0.59545
32	YCR107W	AAD3	1.36231	1.59594	1.75516	2.30492	4.79259
33	YDL022W	GPD1	0.95536	0.88068	1.64979	3.97264	3.5084
34	YDL024C	DIA3	0.88413	0.75221	1.36538	2.46758	3.6631303
35	YDL031W	DBP10	1.61122	2.08052	3.27619	2.20487	1.43297
36	YDL037C		3.0142403	2.70624	0.3301	0.14693	0.47497
37	YDL039C	PRM7	4.11342	4.6858206	0.46122	0.18271	0.12441
38	YDL059C	RAD59	1.27384	1.32102	2.01786	3.62362	6.0652
39	YDL070W	BDF2	1.0365	1.44972	2.60526	2.71815	3.20639
40	YDL075W	RPL31A	1.56944	2.0143	3.0091102	2.4121	0.75203
41	YDL113C		0.97938	0.92197	1.28113	2.10491	3.54966

42	YDL115C		1.0908	1.15849	1.37566	1.99613	3.00302
43	YDL125C	HNT1	1.22652	1.30884	3.05383	4.68391	8.5493
44	YDL169C	UGX2	1.2128	1.28893	2.17714	4.49506	1.66542
45	YDL204W		0.9393	0.73074	1.38918	5.69632	9.932331
46	YDL243C	AAD4	1.45847	1.67523	1.94364	2.48658	4.03121
47	YDR003W		1.04936	1.11417	1.96771	2.83599	3.64743
48	YDR018C		1.08121	1.19178	1.31746	2.47958	4.25155
49	YDR056C		1.15346	1.17336	2.27527	3.2575502	3.2205
50	YDR070C		1.02834	0.86121	1.02096	4.63736	11.93918
51	YDR111C		1.34719	1.391	1.91391	3.1618202	4.73053
52	YDR174W	HMO1	1.1481	1.36264	1.92944	2.88877	3.26418
53	YDR184C	ATC1	2.3198	3.3462002	1.25904	0.76762	0.6323
54	YDR219C		1.14019	1.30057	2.38916	2.91331	3.7844203
55	YDR253C	MET32	0.88377	0.92164	1.01786	1.74309	3.23206
56	YDR256C	CTA1	1.00678	1.3297	1.0625	2.19923	5.23997
57	YDR262W		1.00281	1.13639	1.90785	2.68828	3.6677098
58	YDR306C		1.03452	0.97753	2.19792	3.3892598	4.26821
59	YDR336W		1.46868	1.70028	2.3625	3.4174497	1.51793
60	YDR346C		1.48413	2.06148	3.6093798	3.1203103	2.72309
61	YDR387C		0.78635	0.72793	1.51411	2.63446	3.0599303
62	YDR398W		2.50124	3.5828202	2.09428	0.81503	0.47692
63	YDR435C	PPM1	1.19833	1.26417	1.83004	2.43209	3.35998
64	YDR453C		1.02314	0.89832	1.0219	3.6123	4.25316
65	YDR471W	RPL27B	1.50493	2.08128	3.1332302	1.31769	0.42956
66	YDR492W		2.58023	6.62935	13.0743	10.20106	5.89573
67	YDR496C		1.93937	3.0013602	3.0263703	1.37132	0.60135
68	YDR504C		1.00437	1.03392	1.58559	2.33226	3.02352
69	YDR516C		0.81438	0.69661	1.57257	2.9018703	3.2232897
70	YDR530C	APA2	1.02962	1.13114	1.66489	3.4215798	3.88631
71	YDR542W		0.92785	1.2273	1.625	3.1067197	2.69385
72	YEL011W	GLC3	0.96412	0.75124	2.88229	7.0288205	11.758249
73	YEL039C	CYC7	0.92893	0.73858	1.17658	3.8259	3.18927
74	YEL072W		1.19934	1.57944	4.93791	8.34776	12.152559
75	YER020W	GPA2	0.95994	1.08487	2.46207	3.864	2.30808
76	YER042W	MXR1	1.35873	1.35649	1.62658	2.16199	3.4200802
77	YER053C		0.99461	0.85507	1.66765	3.3705401	4.5278196
78	YER056C	FCY2	2.64863	3.2977998	1.35123	0.32066	0.22148
79	YER065C	ICL1	1.17778	1.66126	2.44286	2.98419	4.15741
80	YER066W		0.78102	0.87729	2.92213	4.50196	4.60095
81	YER067W		0.50699	0.62875	3.71825	7.8568697	8.223431
82	YER078C		1.03939	1.09121	2.2375	3.31545	1.94628
83	YER079W		0.85437	0.82674	1.50259	3.6082	1.96935
84	YER117W	RPL23B	1.51201	1.94439	3.17666	2.00212	0.83311
85	YER150W	SPI1	0.84877	0.77781	1.29897	2.80709	3.0019
86	YFL014W	HSP12	1.20927	0.9725	0.87215	2.46087	9.3936205
87	YFL030W		1.13982	1.11851	1.65561	2.55246	3.8397
88	YFL055W	AGP3	1.00927	1.30234	4.47706	10.714099	18.273357
89	YFL056C	AAD6	1.16193	1.32224	1.62179	2.41718	3.5401

90	YFL057C		1.31235	1.54697	1.78583	2.65886	4.9862905
91	YFR014C	CMK1	1.23873	1.40431	1.78136	2.79512	4.85549
92	YFR015C	GSY1	0.80209	0.61885	1.56667	3.61418	2.04716
93	YFR017C		0.75176	0.65432	1.81091	3.7296097	4.3214703
94	YFR053C	HXK1	1.0097	1.14979	3.8469803	4.8080006	4.44604
95	YGL029W	CGR1	1.47899	2.80426	3.41014	2.20962	1.07818
96	YGL033W	HOP2	0.88809	0.8757	0.86	1.82706	3.46097
97	YGL045W		0.84499	0.88588	1.675	3.94022	3.7547197
98	YGL075C	MPS2	0.92903	0.96407	1.08609	1.9752	3.1905599
99	YGL122C	NAB2	0.81065	0.98731	2.9251502	3.3459601	4.15031
100	YGL135W	RPL1B	1.54394	1.91542	3.12628	2.31277	1.20963
101	YGL179C	TOS3	0.92586	0.97704	3.6000001	5.87365	4.88168
102	YGL184C	STR3	0.93984	0.96212	1.01517	1.91628	3.90721
103	YGL255W	ZRT1	1.94464	2.8635201	3.5871997	4.1866007	10.74703
104	YGL261C		0.93717	1.04224	1.66667	3.0096	3.47758
105	YGR008C	STF2	1.01723	0.82271	2.60458	6.17081	12.13232
106	YGR043C		1.10852	1.05999	1.22387	4.53298	12.765181
107	YGR053C		0.87555	0.85445	1.38938	1.89862	3.0796297
108	YGR088W	CTT1	0.73144	0.5275	2.61392	8.44166	9.138019
109	YGR102C		1.02841	1.10224	0.92	1.99894	3.01003
110	YGR154C		1.08783	1.16426	1.53119	2.40742	4.68076
111	YGR197C	SNG1	1.00376	1.1192	2.2019	3.2474	3.1514597
112	YGR222W	PET54	1.26054	1.42349	1.60366	2.08335	3.08212
113	YGR223C		1.19349	1.26046	2.53929	3.2797003	3.1076899
114	YGR251W		1.6456	2.2153	2.10687	1.95891	3.09891
115	YGR256W	GND2	1.00473	0.91066	0.80725	1.505	3.26524
116	YGR262C		1.1995	1.26261	1.58052	2.31353	3.66908
117	YGR286C	BIO2	1.27075	1.81795	4.17834	4.07809	2.96402
118	YGR294W		0.95577	1.15384	2.4	3.1283402	1.82326
119	YHL016C	DUR3	0.87458	1.24974	3.37398	1.98922	1.26147
120	YHL021C		0.53181	0.33107	1.4273	3.25184	3.4255702
121	YHL036W	MUP3	0.96214	1.05319	2.00749	2.55455	3.1350303
122	YHL046C		0.88892	1.08159	2.48555	3.8127797	3.1550698
123	YHR066W	SSF1	2.03287	3.06945	1.28049	0.39122	0.42343
124	YHR087W		0.96424	0.85725	1.97701	8.501441	11.07285
125	YHR138C		1.38952	1.42322	3.06207	3.10159	4.42282
126	YHR139C	SPS100	0.98016	1.02298	2.97178	10.10476	17.492609
127	YHR141C	RPL42B	1.67425	2.11846	3.2603197	1.71907	0.50346
128	YHR146W		0.73244	1.10551	1.84584	3.1886997	3.3606603
129	YIL036W	CST6	1.0246	1.11707	2.81361	3.82443	3.8948402
130	YIL045W	PIG2	0.71006	0.52756	1.42746	3.0158298	2.69356
131	YIL069C	RPS24B	1.74011	2.2868	3.1866403	1.75181	0.68099
132	YIL077C		1.10246	1.15074	2.4375	3.40083	2.0907
133	YIL107C	PFK26	0.90527	0.77253	1.14115	2.4408	3.8814597
134	YIL136W	OM45	0.84002	0.71072	0.72702	2.82218	3.83757
135	YIL143C	SSL2	1.12017	1.16916	2.10938	2.7908301	3.29
136	YIL153W	RRD1	0.89555	0.93818	1.50312	2.78051	3.1932
137	YJL132W		0.87519	0.78836	1.25191	1.77312	3.03586

138	YJL155C	FBP26	0.95693	0.91742	1.43721	2.23459	4.2299094
139	YJL223C	PAU1	0.94032	1.07645	2.33929	4.01076	3.37687
140	YJR085C		1.22341	1.13732	1.30019	1.75168	3.03429
141	YJR155W	AAD10	1.11394	1.16776	1.52716	2.03351	3.72837
142	YKL026C	GPX1	1.0308	0.96926	2.38347	4.73596	8.17473
143	YKL070W		1.77472	3.02877	7.3672304	13.586381	14.590239
144	YKL071W		1.36053	1.46881	1.66965	2.03659	3.51212
145	YKL078W		2.37036	3.2950103	1.38636	0.90211	0.7957
146	YKL087C	CYT2	1.08878	1.13993	1.52257	2.52182	4.00504
147	YKL089W	MIF2	0.9988	0.93813	1.48333	3.3537698	3.56049
148	YKL090W		0.95164	1.0171	1.17553	3.4655097	2.18442
149	YKL091C		0.75033	0.54752	1.17633	3.13907	4.35414
150	YKL094W	YJU3	1.11442	1.15337	1.49033	2.88253	5.11229
151	YKL103C	LAP4	1.11797	1.18683	1.50443	2.29227	3.98024
152	YKL125W	RRN3	1.28447	1.13671	1.45736	2.11759	3.15104
153	YKL150W	MCR1	0.98958	0.91165	1.66348	3.1258898	4.36814
154	YKL151C		1.02779	0.87802	1.28317	2.40705	3.64677
155	YKL162C		0.90755	1.10725	1.61719	2.48505	5.47137
156	YKL187C		0.95593	1.04668	6.15217	22.404268	15.74071
157	YKL224C		0.89848	1.12533	2.81176	3.25963	2.08497
158	YKR049C		1.25539	1.2027	1.75	3.07264	2.44532
159	YKR075C		3.0095403	3.27039	0.50806	0.95682	0.91958
160	YKR077W		1.14206	1.71613	1.91304	4.17779	2.21691
161	YKR100C		0.91584	1.08788	1.70127	2.34189	3.0141
162	YLL055W		1.13227	1.26922	3.6136997	3.98156	4.27876
163	YLL056C		0.98863	1.09245	2.02424	4.75816	7.3932605
164	YLR009W		1.60275	2.40353	3.1268404	1.1439	0.41884
165	YLR145W		0.97333	1.2659	1.44364	4.03507	3.76035
166	YLR149C		0.63981	0.81805	1.11078	2.54315	3.70763
167	YLR164W		0.7098	0.64934	1.0303	3.7946599	3.82147
168	YLR251W		0.80386	0.68135	0.7995	1.78338	3.56716
169	YLR252W		0.88886	0.75325	0.93727	1.98089	3.0293598
170	YLR266C		1.12845	1.25712	1.67885	2.21115	3.2613397
171	YLR311C		0.9542	1.0924	1.99216	3.5065703	5.76001
172	YLR312C		1.12083	1.11103	2.60448	4.70583	7.24224
173	YLR327C		0.69919	0.88948	1.2234	3.0811	1.38007
174	YLR413W		2.57574	3.95178	3.42857	1.36255	0.67484
175	YLR421C	RPN13	1.27411	1.25963	1.62864	2.07345	3.52192
176	YML004C	GLO1	1.0562	1.13595	1.15769	2.08466	3.8888502
177	YML128C		1.06312	0.88233	1.19443	3.34315	7.0703206
178	YML131W		1.25133	1.36287	1.6693	2.30028	4.31475
179	YMR030W		0.71059	0.81793	1.29167	3.37439	1.48566
180	YMR090W		1.25887	1.12985	1.38467	2.31588	3.95838
181	YMR100W	MUB1	0.98043	1.16973	2.1046	2.71536	3.0512598
182	YMR105C	PGM2	0.93366	0.67889	0.84615	2.33853	3.23185
183	YMR107W		1.05274	0.9975	3.35795	13.346421	26.729939
184	YMR139W	RIM11	0.84478	0.8269	1.88423	2.86575	3.3071597
185	YMR246W	FAA4	1.51515	2.70731	7.9028206	5.6360803	2.22328

186	YMR255W	GFD1	0.86952	1.19143	1.6185	3.07659	1.97355
187	YMR258C		0.83821	0.75127	1.81532	2.99214	3.62522
188	YMR262W		0.91145	0.88096	1.56184	3.65197	3.4286199
189	YMR271C	URA10	1.18771	1.17152	1.64009	3.0692298	6.2542396
190	YMR316W	DIA1	1.14344	1.59805	3.15714	2.4773	3.35398
191	YMR320W		1.01219	1.3638	1.81944	3.53727	1.9338
192	YMR322C		1.07036	0.92294	1.04425	3.2590702	3.9411802
193	YNL011C		0.78709	0.83233	1.16337	2.48838	3.23587
194	YNL024C		1.46682	2.16135	4.9626203	6.2175603	3.3494
195	YNL112W	DBP2	4.01042	6.8630104	6.7637796	3.18022	0.77863
196	YNL117W	MLS1	1.01106	1.25314	1.05263	1.76615	3.1688
197	YNL124W		1.88126	3.18683	1.20395	0.32775	0.43034
198	YNL141W	AAH1	3.2322798	4.3802	2.65306	0.76989	0.63074
199	YNL142W	MEP2	1.11873	1.76151	3.07634	1.71723	1.73964
200	YNL178W	RPS3	1.86694	2.28552	3.26319	2.9932404	1.222
201	YNL194C		0.60936	0.26556	0.54245	3.3947198	2.92208
202	YNL195C		0.77047	0.53543	0.54603	2.8285697	3.674
203	YNL213C		1.21604	1.28292	1.8547	2.64103	3.2764103
204	YNL244C	SUI1	1.42833	1.67499	2.85473	3.05486	3.30479
205	YNL331C	AAD14	1.27426	1.28444	1.46592	1.74846	3.3124697
206	YNR039C	ZRG17	1.35281	1.43951	1.24797	1.87414	3.47606
207	YNR051C	BRE5	1.02042	1.58095	2.44323	3.06008	3.4274
208	YNR053C		2.43279	3.8915102	2.9575803	0.96136	0.70268
209	YNR071C		1.4138	1.79554	2.05263	3.85069	1.97059
210	YNR075W	COS10	1.01794	1.38808	3.92754	5.2499	3.21607
211	YNR076W	PAU6	0.95206	1.06779	1.9951	3.30685	2.41205
212	YOL002C		1.29616	2.15787	9.937701	10.26189	2.54972
213	YOL016C	CMK2	0.98959	1.09154	2.51295	2.511	3.29924
214	YOL084W	PHM7	0.89374	1.06172	1.34868	3.0450897	2.44176
215	YOL101C		1.45019	2.04909	19.401777	25.94569	5.15477
216	YOL108C	INO4	1.00123	1.1017	2.7381	3.71228	4.51399
217	YOL116W	MSN1	0.85785	0.93227	1.41	2.236	3.92998
218	YOL124C		2.62574	3.8273304	1.82258	0.59246	0.50758
219	YOL127W	RPL25	1.43301	1.79808	3.0211596	2.16872	0.60117
220	YOL132W		1.04835	1.24031	1.54286	3.106	1.38277
221	YOL153C		0.5938	0.52128	1.14079	2.9794703	3.91833
222	YOL154W		1.34726	1.6499	1.56098	3.08407	2.6685
223	YOL161C		0.96664	1.03449	2.15947	3.3816001	2.71004
224	YOL162W		0.96196	1.16852	2.69014	5.84978	2.8762603
225	YOL163W		0.96778	1.11322	2.8315797	5.91772	10.61694
226	YOL165C	AAD15	1.22875	1.30924	1.71266	2.40526	3.41076
227	YOR019W		0.9127	0.89891	1.57317	3.0450897	2.8125703
228	YOR031W	CRS5	0.99219	0.97751	1.31677	2.15347	4.49082
229	YOR043W	WHI2	1.13856	1.49043	2.8123202	2.99276	3.5548503
230	YOR095C	RKI1	2.54298	3.30516	2.6587	0.74247	0.51106
231	YOR292C		0.8462	0.8146	1.8877	3.63797	3.36628
232	YOR298W		1.13806	1.34351	2.20339	3.03402	1.37237
233	YOR391C		1.02427	0.93243	1.21622	3.1344903	4.50622

234	YOR394W		0.93912	1.01044	2.27089	3.1992402	3.01734
235	YPL004C		0.96002	0.89191	1.30196	2.85464	4.09551
236	YPL014W		0.52817	0.7035	3.98544	5.42407	2.79718
237	YPL015C	HST2	0.9054	0.99103	1.71743	2.89551	3.0497203
238	YPL043W	NOP4	1.46609	2.46296	3.48128	1.46577	0.92248
239	YPL054W	LEE1	1.2276	1.48657	2.8826299	4.24342	4.39311
240	YPL093W	NOG1	2.31796	3.42804	2.76864	0.35724	0.21256
241	YPL107W		1.20868	1.27572	1.78309	4.46614	4.12846
242	YPL122C	TFB2	1.20815	1.39332	2.20091	3.24515	1.51419
243	YPL149W	APG5	1.24369	1.40163	1.99367	2.35995	3.04209
244	YPL171C	OYE3	1.23205	1.16524	1.72912	2.60267	3.9988701
245	YPL186C		0.93532	0.73402	1.39045	4.42236	7.3833003
246	YPL223C	GRE1	1.02493	0.95208	0.96131	4.45988	19.046879
247	YPL224C	MMT2	1.10505	1.2202	1.81892	2.67785	3.53972
248	YPL245W		1.25743	1.27819	1.94444	3.15968	0.71742
249	YPL250C	ICY2	1.35039	1.62825	1.53159	1.8243	3.9844902
250	YPL280W		1.00028	0.8794	1.08612	3.5273502	6.96237
251	YPL281C	ERR2	0.85369	0.76881	0.77273	1.15636	3.3315897
252	YPL282C		0.94257	1.01298	2.54118	3.04157	2.20817
253	YPR045C		0.90533	0.81637	1.32194	2.57976	3.18647
254	YPR061C		1.28251	1.48397	2.21667	2.86721	3.37915
255	YPR086W	SUA7	1.33226	1.38649	2.12824	2.46114	3.1201
256	YPR121W	THI22	0.85368	1.03169	2.96296	4.04236	2.568
257	YPR143W		1.24535	1.88597	3.25366	2.03536	1.03127
258	YPR160W	GPH1	0.93853	0.73339	1.57334	3.6765997	3.65112
259	YPR200C	ARR2	1.19842	1.10383	1.30505	1.7543	3.19482

Table 4 shows: systematic gene names of yeasts; common names (only in a case where such a common name is given) (wherein, with regard to these gene names and common names, please refer to the yeast genome database (*Saccharomyces cerevisiae* genome database; http://genome-www.stanford.edu/Saccharomyces/); and the ratios of the normalized values of fluorescence intensities of post-low temperature treatment samples at various periods of time to the normalized values of fluorescence intensities of pre-low temperature treatment samples.

Example 2 Confirmation of cold inducibility of cold-inducible gene

In order to confirm the cold inducibility of each cold-inducible gene identified by DNA microarray analysis, Northern blotting analysis was carried out according to the method described in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory. In order to measure the amount of RNA by Northern blotting analysis, probe DNA used to specifically

detect RNA of interest was first prepared by the polymerase chain reaction (PCR) method. In the preset example, a method of producing the DNA probe of YFL014W (HSP12), one of the cold-inducible genes identified in Example 1, will be specifically described. Using the genome DNA of a *Saccharomyces cerevisiae* YPH500 strain, and an HSP12-F primer and an HSP12-R primer complementary to nucleotide sequences in the ORF of an HSP12 gene, and applying Expand High Fidelity PCR system (Roche), an HSP12 fragment consisting of approximately 330 bases was amplified with Takara PCR Thermal Cycler MP in accordance with the manual attached therewith.

The sequences of the above primers are as follows. With regard to the positions of the primers, please refer to the above-described yeast genome database.

HSP12-F: ATGTCTGACGCAGGTAGAAAAG (SEQ ID NO: 1)

HSP12-R: TTACTTCTTGGTTGGGTCTTCTTC (SEQ ID NO: 2)

PCR was carried out using 100 μl of a reaction solution containing 300 nM each primer, 200 μM dNTP (a mixed solution consisting of 4 types of deoxynucleotide triphosphate), 100 ng of the genome DNA of the *Saccharomyces cerevisiae* YPH500 strain, and a buffer (1 x) and 2.6 U Expand HiFi DNA polymerase attached with the Expand High Fidelity PCR system, under conditions consisting of: a first step of 95°C, 2 minutes; a second step of 35 cycles consisting of 95°C, 30 seconds (denaturation), 55°C, 30 seconds (annealing), and 72°C, 1 minute (elongation); and a third step of 72°C, 5 minutes.

Subsequently, the prepared HSP12 fragment was ligated to a pT7Blue T-vector (Novagen), and *Escherichia coli* DH5α was transformed with the obtained vector. Several transformants were cultured in a test tube, and a plasmid was then prepared using QuantumPrep Plasmid MiniPrep kit (Bio-Rad). Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. Thereafter, the obtained transformant was cultured in 80 ml of a culture solution, and a plasmid was prepared using QuantumPrep Plasmid MidiPrep kit (Bio-Rad). The nucleotide sequence of the obtained HSP12 fragment was sequenced using DNA sequencing kit (Applied Biosystems), and the obtained nucleotide sequence of the HSP12 fragment was compared with the nucleotide sequence of HSP12 in the genome database (*Saccharomyces cerevisiae* genome

database; http://genome-www.stanford.edu/Saccharomyces/), so as to identify it. Thereafter, an HSP12 fragment was cut out of the pT7Blue T-vector containing the HSP12 fragment, using restriction enzymes. The HSP12 fragment was then separated and recovered by agarose gel electrophoresis using low melting point agarose (FMC). The thus obtained HSP12 fragment was labeled with alkaline phosphatase, using AlkPhos Direct Labeling Module (Amersham Biotech) in accordance with the protocol attached therewith.

Likewise, with regard to YNL112W (DBP2), YGR159C (NSR1), YNL141W (AAH1), YKR075C, YGL055W (OLE1), and YFL039C (ACT1), the same above operations were carried out using primers complementary to nucleotide sequences in ORF, so as to produce a probe used in Northern blotting.

The sequences of primers are as follows.

Primers to DBP2

DBP2-F: GGATGACTTACGGTGGTAGAGATC (SEQ ID NO: 3)

DBP2-R: AAGATACCTCTGGCGGCCAC (SEQ ID NO: 4)

Primers to NSR1

NSR1-F: GGTAACAAGAAGGAAGTTAAGGCTTC (SEQ ID NO: 5)

NSR1-R: TGTTTTCTTTGAACCAGCGAAAG (SEQ ID NO: 6)

Primers to AAH1

AAH1-F: GGTTTCTGTGGAGTTTTTACAGGAG (SEQ ID NO: 7)

AAH1-R: GCGAATATTTAGTGACTACTTCGTCC (SEQ ID NO: 8)

Primers to YKR075C

YKR075C-F: TGGACGATACAATAATTTCGTACCA (SEQ ID NO: 9)

YKR075C-R: CAACCTGGTTCCTATAAAAAATGTCTT (SEQ ID NO: 10)

Primers to OLE1

OLE1-F: GGAAGCTTATGCCAACTTCTGGAACTACTATT (SEQ ID NO: 11)

OLE1-R: GGAAGCTTTTAAAAGAACTTACCAGTTTCGTAG (SEQ ID NO: 12)

Primers to ACT1

ACT1-F: TCAAAAAGACTCCTACGTTGGTGATGAAGC (SEQ ID NO: 13)

ACT1-R: CATACGCGCACAAAAGCAGAGATTAGAAAC (SEQ ID NO: 14)

For NSR1, AAH1, and YKR075, PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment. For DBP2, PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment with the exception that the annealing temperature in the second step was changed from 55°C to 47°C and that the elongation reaction time (72°C) was changed from 1 minute to 1.5 minutes. For OLE1 and ACT1, PCR was carried out using 100 μl of a reaction solution containing 200 nM each primer, 200 μM dNTP, 1 μg of the genome DNA of the *Saccharomyces cerevisiae* YPH500 strain, and a 1 x natural Pfu polymerase buffer (Stratagene) and 2.5 U Pfu DNA polymerase, under conditions consisting of: a first step of 94°C, 2 minutes; a second step of 25 cycles consisting of 94°C, 30 seconds (denaturation), 55°C, 30 seconds (annealing), and 72°C, 3 minutes (elongation); and a third step of 72°C, 5 minutes. It is to be noted that with regard to OLE1 and ACT1, DNA amplified by PCR was not phosphorylated, but directly subcloned into a pZErO2 vector (Invitrogen) that had previously been cleaved with *Eco*RV.

Subsequently, 10 μg of RNA prepared in the same manner as in Example 1 was subjected to 1% denatured agarose gel electrophoresis, and RNA was then transferred to Hybond-N+ (Amersham Biotech) overnight. The obtained filter was hybridized with the labeled HSP12 fragment as prepared above in accordance with the protocol of AlkPhos Direct Labeling Module. Thereafter, using CDP-Star Detection Reagent (Amersham Biotech), the hybridized HSP12 mRNA was detected by exposure to an X-ray film, and then assayed. Likewise, using ECF Detection Module, the concentration of the purified fluorescent substance was detected with Molecular Imager FX Pro (Bio-Rad), and then assayed. The results are shown in Figure 1. From Figure 1, it became clear that the amount of HSP12 mRNA increased from 4 hours after the culture temperature was decreased to 10°C. Likewise, the results of Northern blotting analysis performed on DBP2, NSR1, AAH1, YKR075C, OLE1, and ACT1 are shown in Figures 2, 3, 4, 5, 6, and 7, respectively. In the case of DBP2, NSR1, AAH1, and YKR075C, the amount of mRNA became greater than that of a pre-low temperature treatment sample at 1 hour after the culture temperature was decreased to 10°C. In the case of OLE1 also, an increase in the amount of mRNA by a low temperature treatment was observed, and the amount of mRNA reached the maximum at 2

hours after a decrease in the culture temperature. When Northern blotting analysis was carried out on ACT1 as a negative control, the amount of mRNA of which had not been changed by a low temperature treatment in the DNA microarray, it was found that the amount of the mRNA was hardly changed by such a low temperature treatment (Figure 7). From these results, in all the cases of HSP12, DBP2, NSR1, AAH1, YKR075C, and OLE1, an increase in the amount of mRNA by a low temperature treatment, obtained by the DNA microarray, could be confirmed by Northern blotting analysis. Accordingly, with regard to these genes, it was found that a promoter for increasing the amount of mRNA in response to a low temperature exists in the non-translation region located upstream of the 5'-terminal side of each gene.

Example 3 Cold induction of DNA sequence located downstream by DNA fragment having cold-inducible promoter function

A DNA fragment having a cold-inducible promoter function was isolated, and heterogenous DNA was ligated downstream thereof, so that the production of RNA from DNA located downstream could be induced by a low temperature treatment. This was confirmed as follows. First, a DNA fragment having a DBP2 cold-inducible promoter function was isolated. The 5' upstream adjacent gene of DBP2 is YNL113W (RPC19). A region sandwiched between RPC19 and DBP2 (that is, a non-translation region located upstream of the 5'-terminal side of DBP2) was isolated by PCR, using two primers, each consisting of 24 bases located downstream of the 3'-terminal side adjacent to the ORF of RPC19 (RPC19-DBP2 IGR F) and 28 bases located upstream of the 5'-terminal side adjacent to the ORF of DBP2 (RPC19-DBP2 IGR R), and the genome DNA of a *Saccharomyces cerevisiae* YPH500 strain.

The sequences of the primers are as follows.

RPC19-DBP2 IGR F: ATGTTACGGATCGACTCAAAGACC (SEQ ID NO: 15)

RPC19-DBP2 IGR R: ATTTGCTCTAAATTTGCCTTAATAGTGC (SEQ ID NO: 16)

PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment.

Subsequently, the isolated DNA was inserted into the site located upstream of the ORF of an enhanced green fluorescent protein (EGFP) in a reporter plasmid pUG35-MET25. It is to be noted that the pUG35-MET25 plasmid was produced by cleaving pUG35 (http://www.mips.biochem.mpg.de/proj/yeast/info/tools/index.html) with *Xba*I and *Sac*I, and blunt-ending the cleaved portion with T4 DNA polymerase, followed by the self-cyclization of the obtained product. The pUG35-MET25 plasmid was cleaved with *Sal*I, and then converted into a blunt end with T4 DNA polymerase. Thereafter, hydroxyl groups at both ends of the DNA fragment having a DBP2 cold-inducible promoter function, which had been isolated by PCR, were phosphorylated with T4 DNA kinase and ATP. The phosphorylated DNA fragment having a DBP2 promoter function was ligated to the blunt-ended pUG35-MET25 plasmid, using TaKaRa DNA Ligation Kit ver. 2 in accordance with the protocol attached with the kit. Thereafter, *Escherichia coli* DH5α was transformed with the ligated product. Several transformants as obtained above were cultured in 3 ml of a culture solution overnight, and plasmids were then prepared using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. In addition, at this time, a plasmid in which a DBP2 promoter is adjacent upstream of EGFP ORF (forward direction; Figure 8), and a plasmid in which a region adjacent to an RPC19 side is ligated immediately upstream of EGFP ORF (reverse direction), were isolated. Thereafter, a transformant obtained in each case was cultured in 80 ml of a culture solution, and a plasmid was then prepared using QuantumPrep Plasmid MidiPrep kit. A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with this plasmid. Transformation was carried out by the method described in Yeast Protocol Handbook published from Invitrogen. The obtained transformed yeast was cultured at 30°C, and at the time when the absorbance at 600 nm became 1, sampling was carried out at 0 minute. Thereafter, the culture temperature was decreased from 30°C to 10°C, and the culture was continuously carried out. Then, sampling was carried out in the same manner as described above.

Using these samples, RNA was prepared from yeast by the same method as in Example 2, and the amount of EGFP mRNA was measured by Northern blotting analysis. A probe

used in Northern blotting analysis was produced by cleaving pGFPuv (Clontech) with restriction enzymes *Pst*I and *Eco*RI and recovering a GFP fragment. Northern blotting analysis was carried out by the same method as in Example 2. The results are shown in Figure 9. From Figure 9, it was proved that the use of a DNA fragment having a DBP2 cold-inducible promoter function promotes the transcription of DNA located downstream thereof by a low temperature treatment. Such cold inducibility was not observed, when the DNA fragment having a DBP2 cold-inducible promoter function was inserted into pUG35-MET25 in the reverse direction (Figure 10). Thus, it was confirmed that transcriptional activation by a low temperature shown in Figure 9 is caused by the function of a DBP2 promoter.

Likewise, with regard to DNA fragments having functions of cold-inducible promoters of YBR034C (HMT1) and YFL014W (HSP12), which were identified as cold-inducible genes in Example 1, their cold inducibility was confirmed.

First, as with the above DBP2, a DNA fragment having an HMT1 cold-inducible promoter function was isolated. The 5' upstream adjacent gene of HMT1 is YBR035C (PDX3). A region sandwiched between PDX3 and HMT1 (that is, a non-translation region located upstream of the 5'-terminal side of HMT1) was isolated by PCR, using two primers, each consisting of 25 bases located downstream of the 3'-terminal side adjacent to the ORF of PDX3 (PDX3-HMT1 IGR F) and 25 bases located upstream of the 5'-terminal side adjacent to the ORF of HMT1 (PDX3-HMT1 IGR R).

The sequences of the primers are as follows.

PDX3-HMT1 IGR F: GGGACTGTTAATGAAAAATTCAATG (SEQ ID NO: 17)

PDX3-HMT1 IGR R: TATTTTCTTTGGATGAATTTGTCGG (SEQ ID NO: 18)

PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment with the exception that the annealing temperature was changed from 55°C to 50°C in the second step.

A DNA fragment having an HMT1 cold-inducible promoter function, which was obtained by the same method as in the case of the above DNA fragment having a DBP2 cold-inducible promoter function, was inserted into pUG35-MET25 (this time, a product in

which the DNA fragment was inserted therein in the reverse direction was also prepared). Thereafter, cold inducibility was confirmed in the same manner as described above, using an increase in the amount of EGFP mRNA as an indicator. As a result, when the DNA fragment having an HMT1 cold-inducible promoter function was located immediately upstream of EGFP in a correct direction, cold inducibility could be confirmed (Figure 11). However, when it was inserted therein in the reverse direction, cold inducibility was not observed (Figure 12). From these results, it was found that the use of a DNA fragment having an HMT1 cold-inducible promoter function enables induction of the transcription of DNA located downstream thereof by a low temperature.

Thereafter, a DNA fragment having an HSP12 cold-inducible promoter function was isolated. The 5' upstream adjacent gene of HSP12 is YFL015C. However, since both genes were very close to each other and a coding region existed in the opposite chain of DNA, a region comprising a portion of the YFL015C gene and the sandwiched portion between YFL015C and HSP12 (that is, a non-translation region located upstream of the 5'-terminal side of HSP12), was isolated by PCR, using two primers, each consisting of 19 bases located in the antisense chain in the ORF of YFL015C (-610 HSP12) and 28 bases located upstream of the 5'-terminal side adjacent to the ORF of HSP12 (HSP12 IGR R).

The sequences of the primers are as follows.

-610 HSP12 IGR F: GATCCCACTAACGGCCCAG (SEQ ID NO: 19)

HSP12 IGR R: TGTTGTATTTAGTTTTTTTTGTTTTGAG (SEQ ID NO: 20)

PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment with the exception that the annealing temperature was changed from 55°C to 50°C in the second step.

A DNA fragment having an HSP12 cold-inducible promoter function, which was obtained by the same method as in the case of the above DNA fragment having a DBP2 or HMT1 cold-inducible promoter function, was inserted into pUG35-MET25 (this time, a product in which the DNA fragment was inserted therein in the reverse direction was also prepared). Thereafter, cold inducibility was confirmed in the same manner as described above, using an increase in the amount of EGFP mRNA as an indicator. As a result, when

the DNA fragment having an HSP12 cold-inducible promoter function was located immediately upstream of EGFP in a correct direction, cold inducibility could be confirmed (Figure 13). However, when it was inserted therein in the reverse direction, cold inducibility was not observed (Figure 14). From these results, it was found that the use of a DNA fragment having an HSP12 cold-inducible promoter function enables induction of the transcription of DNA located downstream thereof by a low temperature.

Example 4 Identification of cold-inducible cis sequence

A cis sequence of a DNA fragment having the cold-inducible promoter function of a gene exhibiting cold inducibility at an early stage was identified as follows. First, in the experiment described in Example 1, genes whose signal increased to 2 times or more at 15 minutes after the culture temperature was decreased to 10°C were identified. The identified 41 genes are shown in the following Table 5.

Table 5

Genes exhibiting 2 times or more of cold inducibility after 15 minutes

No.	Systematic gene name	Common name	15 minutes (Normalized)	No.	Systematic gene name	Common name	15 minutes (Normalized)
1	YDL039C	PRM7	4.11342	22	YDL063C		2.16531
2	YNL141W	AAH1	3.2322798	23	YOR360C	PDE2	2.08345
3	YDL037C		3.0142403	24	YHR196W		2.08005
4	YKR075C		3.0095403	25	YNL065W		2.05877
5	YER056C	FCY2	2.64863	26	YHR066W	SSF1	2.03287
6	YOL124C		2.62574	27	YLR407W		2.01923
7	YDR492W		2.58023	28	YOR101W	RAS1	2.00532
8	YLR413W		2.57574	29	YNL112W	DBP2	4.01042
9	YCR072C		2.54439	30	YGR159C	NSR1	2.9673197
10	YOR095C	RKI1	2.54298	31	YGL055W	OLE1	2.51904
11	YNL175C	NOP13	2.5208	32	YNR053C		2.43279
12	YDR398W		2.50124	33	YPL093W	NOG1	2.31796
13	YGR283C		2.40094	34	YHR170W	NMD3	2.08971
14	YBR296C	PHO89	2.39344	35	YHR148W	IMP3	2.04153
15	YDR184C	ATC1	2.3198	36	YBR034C	HMT1	2.63514
16	YOR338W		2.25481	37	YOL010W	RCL1	2.46012
17	YAL025C	MAK16	2.23906	38	YKL078W		2.37036
18	YOR063W	RPL3	2.21192	39	YMR290C	HAS1	2.35991
19	YIL096C		2.19996	40	YDR101C		2.28186

20	YER127W	LCP5	2.19577	41	YBL054W		2.15287
21	YBL042C	FUI1	2.16603				

As with Table 4, Table 5 shows systematic gene names of yeasts, common names (only in a case where such a common name is given), and the ratios of the normalized values of fluorescence intensities of samples after being subjected to a low temperature treatment for 15 minutes to the normalized values of fluorescence intensities of pre-low temperature treatment samples.

Using Gene Spring (Silicon Genetics), cis sequences existing between the ORF of each of the above genes and the site 600 bp upstream thereof were searched. As a result, cis sequences could be obtained as DNA sequences that were common in some of these genes.

The cis sequences are as follows.

(a) DNA sequence A: GCTCATCG

(b) DNA sequence B: GAGATGAG

Specifically, the above DNA sequence A was found as a cis sequence that was common in YNL112W (DBP2), YGR159C (NSR1), YGL055W (OLE1), YNR053C, YPL093W (NOG1), YHR170W (NMD3), and YHR148W (IMP3) (which correspond to Nos. 29 to 35 in Table 5), and the above DNA sequence B was found as a cis sequence that was common in YBR034C (HMT1), YOL010W (RCL1), YKL078W, YMR290C (HAS1), YDR101C, and YBL054W (which correspond to Nos. 36 to 41 in Table 5).

Example 5 Confirmation of cold inducibility of cold-inducible cis sequences

In order to confirm that the DNA sequence A (GCTCATCG) obtained Example 4 has cold inducibility, the DNA sequence A was removed from a DNA fragment with a DBP2 cold-inducible promoter function having the above sequence, so as to confirm whether or not the cold inducibility was lost. First, the DNA fragment having a DBP2 cold-inducible promoter function prepared by PCR in Example 3 was ligated to a pT7Blue T-vector, using TaKaRa DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the obtained vector. A plasmid was prepared from the obtained transformant, and it was then

sequenced, so as to confirm its nucleotide sequence. Subsequently, the plasmid as a whole, excluding the DNA sequence A, was amplified by Inverse PCR using outward primers complementary to sequences located at both ends of the DNA sequence A in the plasmid (see Figure 8).

The sequences of the primers are as follows.

RPC19-DBP2 IGR-cis F: CAGAAAATTTTTCCTTCAGTTTATTTG (SEQ ID NO: 21)

RPC19-DBP2 IGR-cis R: ATCGGCGTAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO: 22)

PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment with the exception that the annealing temperature was changed from 55°C to 50°C and the elongation reaction time (72°C) was changed from 1 minute to 5 minutes in the second step, and that the reaction time was changed from 5 minutes to 10 minutes in the third step.

The amplified DNA was subjected to self-circularization using TaKaRa DNA Ligation Kit ver. 2, and *Escherichia coli* DH5α was then transformed again with the obtained vector. Thereafter, plasmid DNA was prepared from several transformants as obtained above, and the nucleotide sequence thereof was determined. Thus, a clone was identified, from which only the DNA sequence A was removed but other nucleotide sequence portions of the DNA fragment having a DBP2 cold-inducible promoter function were not changed. Thereafter, using such a modified clone as a template, a DNA fragment having a modified DBP2 cold-inducible promoter function that was modified by the same method as in Example 3 was amplified by PCR. The amplified DNA fragment was phosphorylated, and then inserted into pUG35-MET25, so as to produce a reporter plasmid. A yeast strain, *Saccharomyces cerevisiae*, was transformed with this reporter plasmid, and samples were then prepared in the same manner as in Example 3, followed by performing Northern blotting analysis. The results are shown in Figure 15. As shown in Figure 15, when compared with the case where a native DNA fragment having a DBP2 cold-inducible promoter function was ligated upstream of EGFP DNA (Figure 15, +cis), cold inducibility became weak by removing the DNA

sequence A from the above DNA fragment (Figure 15, –cis). Thus, it could be confirmed that it was a cis sequence in which the DNA sequence A was associated with cold induction.

Likewise, the DNA sequence B (GAGATGAG) was removed from a DNA fragment with an HMT1 cold-inducible promoter function having the above sequence, so as to confirm whether or not the cold inducibility of the DNA fragment with an HMT1 cold-inducible promoter function was lost. First, the DNA fragment having an HMT1 cold-inducible promoter function was inserted into a pT7Blue T-vector by the same method as in the case of the DNA sequence A. Then, Inverse PCR was carried out using outward primers complementary to sequences located at both ends of the DNA sequence B in the plasmid. Thereafter, the same above analysis was carried out.

The sequences of the primers are as follows.

PDX3-HMT1 IGR-cis F: AACAACTATTTTTATAACATATAATTTCCC (SEQ ID NO: 23)

PDX3-HMT1 IGR-cis R: CTGCCTACTGCTCACCTTG (SEQ ID NO: 24)

PCR was carried out under the same conditions as in the above described PCR for removing a cis sequence from the non-translation region located upstream of the 5'-terminal side of DBP2.

The results of Northern blotting analysis are shown in Figure 16. When compared with the case where a native DNA fragment having a HMT1 cold-inducible promoter function was ligated upstream of EGFP DNA (Figure 16, +cis), cold inducibility was lost by removing the DNA sequence B from the above DNA fragment (Figure 16, –cis). Thus, it was confirmed that it was a cis sequence in which the DNA sequence B was associated with cold induction.

Example 6 Expression of protein by DNA fragment having cold-inducible promoter function, and comparison with other yeast promoters

Using a DNA fragment having a cold-inducible promoter function, it was confirmed that the DNA fragment allows a foreign gene ligated downstream thereof to express. In addition, in order to demonstrate the usefulness as an expression system, a cold-inducible promoter was compared with known promoters. Specifically, a DNA fragment having an

HSP12 cold-inducible promoter function was compared with an alcohol dehydrogenase (ADH1) promoter and a glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter. 3 types of expression vectors having the same plasmid structure were produced as follows, and compared.

As a plasmid comprising the DNA fragment having an HSP12 cold-inducible promoter function, the plasmid described in Example 3 was used.

A plasmid comprising an ADH1 promoter was produced as follows. First, a yeast expression vector pAAH5 having an ADH1 promoter (provided from Dr. Ryo Sato, an emeritus professor of Osaka University; Methods Enzymol. 101, 192-201 (1983)) was cleaved with *Sph*I and *Hind*III. The cleaved portion was then blunt-ended with DNA Blunting Kit (Takara). Thereafter, the DNA fragment was fractionated by agarose gel electrophoresis, so as to recover a fragment containing the ADH1 promoter (approximately 400 bp). On the other hand, as in the case of the DNA fragment having an HSP12 cold-inducible promoter function, a plasmid pUG35-MET25 was cleaved with *Sal*I, and the cleaved portion was then blunt-ended with DNA Blunting Kit, followed by performing dephosphorization with bacterial alkaline phosphatase. The above fragment containing an ADH1 promoter was ligated to the plasmid pUG35-MET25 using DNA Ligation Kit ver. 2 (Takara). Thereafter, *Escherichia coli* DH5α was transformed with the ligated product. The obtained transformant was cultured overnight. Thereafter, a plasmid was extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant containing a plasmid of interest was distinguished. From this transformant, an expression plasmid having an ADH1 promoter was prepared.

A plasmid comprising a TDH3 promoter was produced as follows. First, a yeast expression vector pG-3 having a TDH3 promoter (provided from Dr. Tadashi Nagashima of Shin Nihon Chemical Co., Ltd.; Methods Enzymol. 194, 389-398 (1991)) was cleaved with *Bam*HI and *Hind*III. The cleaved portion was then blunt-ended with DNA Blunting Kit. Thereafter, the DNA fragment was fractionated by agarose gel electrophoresis, so as to recover a fragment containing the TDH3 promoter (approximately 660 bp). The obtained DNA fragment was inserted into the *Sal*I site of a plasmid pUG35-MET25 by the same

method as described above. A transformant having a plasmid with a structure of interest was selected, and finally, an expression plasmid having a TDH3 promoter was prepared.

These 3 types of plasmids had the same structure other than their promoters. A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with each of these 3 types of plasmids. The obtained transformed yeast was inoculated into a synthetic medium containing no uracil (0.67% yeast nitrogen base (containing no amino acid), 2% glucose, 0.02 mg/ml adenine sulfate, 0.02 mg/ml tryptophan, 0.02 mg/ml histidine, 0.03 mg/ml leucine, and 0.03 mg/ml lysine), followed by performing a shake culture at 30°C. With regard to yeast transformed with an expression plasmid comprising an ADH1 promoter and yeast transformed with an expression plasmid comprising a TDH3 promoter, a culture solution thereof was recovered at the time when the absorbance at 600 nm became approximately 1.3. With regard to yeast transformed with an expression plasmid comprising a DNA fragment having an HSP12 cold-inducible promoter function, a culture solution thereof contained in a flask was immersed in a water bath that had previously been set at 10°C, at the time when the absorbance at 600 nm became 0.5. Thereafter, while the flask was gently shaken for 15 minutes, it was quenched. The flask was then transferred into a low temperature thermostat that had previously been set at 10°C, and a shake culture was continued at 10°C. The time when the culture solution was immersed in a water bath at 10°C was determined at 0 minute, and sampling was carried out over time. Extraction of RNA from yeast was carried out in the same manner as in Example 1. Ten μg of the prepared RNA was subjected to Northern blotting analysis by the method described in Example 2. The results are shown in Figure 17.

The middle case in Figure 17 shows the amount of EGFP mRNA obtained when the culture temperature for yeast transformed with 3 types of plasmids was 30°C, or when the temperature was decreased from 30°C to 10°C. The amount of EGFP mRNA produced from the ADH1 promoter and TDH3 promoter, which are commonly used at 30°C, was compared with the amount of EGFP mRNA produced from a DNA fragment having an HSP12 cold-inducible promoter function in yeast wherein the temperature was decreased from 30°C to 10°C. The obtained results are shown over time. The lower case in Figure 17 shows the results obtained by comparing the amount of EGFP mRNA produced from a TDH 3 promoter,

which was found to be relatively stronger than ADH1 promoter from the results of the middle case, with the amount of EGFP mRNA produced from a DNA fragment having an HSP12 cold-inducible promoter function in yeast, at 30°C, or when the temperature was decreased from 30°C to 10°C, over time.

From these results, it was found that a higher EGFP mRNA level was obtained when a DNA fragment having an HSP12 cold-inducible promoter function was used, than when known promoters such as an ADH1 promoter or TDH3 promoter were used.

Subsequently, the TDH3 promoter showing a higher mRNA level than that of the ADH1 promoter was used as a control, and it was compared with the DNA fragment having an HSP12 cold-inducible promoter function in terms of a protein production level. Sampling was carried out in the same manner as described above. After completion of the sampling, yeast recovered by centrifugation was added in the presence of 5 mM DTT using CelLytic™ Y (Sigma) and Protease Inhibitor Cocktail (Sigma), such that it had a concentration described in the manual attached with each of the above instruments. It was then vigorously vortexed at 4°C for 1 hour. Subsequently, the solution was centrifuged at 4°C at 15,000 rpm for 10 minutes. Thereafter, the supernatant was used as a total protein extract in the subsequent analysis. Thirty μg of the total protein extract was subjected to SDS-PAGE (12.5% gel) according to a common method (described in *Tanpakushitsu Jikken Note*, edited by Masato Okada and Kaori Miyazaki, Yodosha Co., Ltd., etc.). Thereafter, a protein separated by the method described in the manual was transferred to Immobilon-P (Millipore). Thereafter, using a 1,000 times diluted anti-GFP antibody (Living Colors™ A.v. Peptide Antibody, Clontech) and ECL PLUS Western Blotting Detection Kit (Amersham Biosciences), Western blotting analysis was carried out in accordance with the manual attached with each instrument, so as to detect an EGFP protein. The results are shown in Figure 18.

The lower case in Figure 18 shows the amount of the EGFP protein over time, which was obtained when the culture temperature for yeast transformed with a plasmid comprising a TDH3 promoter, or DNA fragment having an HSP12 cold-inducible promoter function, was decreased from 30°C to 10°C.

From these results, it was found that a larger amount of protein could be produced when it was inducibly produced at 10°C using a DNA fragment having an HSP12 cold-inducible promoter function, than when it was produced at 30°C using the existing TDH3 promoter.

Example 7 Construction of other expression vectors comprising DNA fragment having cold-inducible promoter function, and expression of protein by other types of yeast (*Saccharomyces cerevisiae*) strains

First, various plasmids were produced by incorporating various types of restriction sites into the positions before and after EGFP. At first, using a plasmid pUG35-MET25, the ORF of EGFP was amplified by PCR.

The sequences of the used primers are as follows.

EGFP3 ORF F: ATGTCTAAAGGTGAAGAATTATTCACTGG (SEQ ID NO: 25)

EGFP3 ORF R: TTATTTGTACAATTCATCCATACCATGGG (SEQ ID NO: 26)

EGFP3 ORF F corresponded to a 29-bp downstream portion including an EGFP initiation codon ATG in the plasmid pUG35-MET25 used in Example 3. EGFP3 ORF R was a sequence complementary to a 29-bp upstream portion including an EGFP termination codon in the same above plasmid.

PCR was carried out under the same conditions as in the above amplification of the HSP12 fragment in Example 2 with the exception that 1 ng of a plasmid pUG35 was used, that the annealing temperature was set at 50°C, and that 30 cycles of reactions were carried out. The amplified DNA was phosphorylated with T4 polynucleotide kinase (Takara). On the other hand, pYES2 (purchased from Invitrogen) was cleaved with *Eco*RI, and the cleaved portion was then blunt-ended with DNA Blunting Kit, followed by performing dephosphorization with bacterial alkaline phosphatase. The amplified EGFP ORF was ligated to the blunt-ended pYES2 using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a

plasmid of interest was identified. A plasmid pYES2+EGFP3 was prepared from this transformant.

Subsequently, in order to produce a plasmid having a centromere as a replication origin, the plasmid pYES2+EGFP3 was cleaved with *Hpa*I and *Mlu*I, so as to recover a DNA fragment having a size of approximately 450 bp. On the other hand, pUG35-MET25 comprising the DNA fragment having an HSP12 cold-inducible promoter function produced in Example 3 (hereinafter referred to as pUG35+PHSP12) was also cleaved with *Hpa*I and *Mlu*I. Thereafter, the above approx. 450-bp DNA fragment was ligated to the pUG35+PHSP12 (approximately 6 kb) using DNA ligation kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pUG35+PHSP12+MCS was prepared from this transformant.

Moreover, in order to produce a plasmid having 2 μ as a replication origin, the obtained plasmid pUG35+PHSP12+MCS was cleaved with *Spe*I and *Mlu*I. The cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate and recover an expression unit (approximately 1.6 kb). On the other hand, pYES2 was also cleaved with *Spe*I and *Mlu*I, and the cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate and recover a pYES2 vector fragment (approximately 5.1 kb). The above expression unit was ligated to the pYES2 vector fragment using Ligation Kit ver. 2, and *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pYES2+PHSP12+EGFP3 was prepared from this transformant.

Furthermore, in order to produce a plasmid having 2 μ as a replication origin and having a weak leucine synthetase gene (leu2-d), the obtained plasmid pUG35+PHSP12+MCS was cleaved with *Hind*III and *Kpn*I. The cleaved portion was subjected to agarose gel electrophoresis, so as to obtain a DNA fragment (approximately 1.7 kb) containing an EGFP3

expression unit. On the other hand, pYEX-BX (purchased from AMRAD Biotech) was also cleaved with *Hind*III and *Kpn*I, and the cleaved portion was subjected to agarose gel electrophoresis, so as to recover a pYEX-BX vector fragment (approximately 6.3 kb). The above DNA fragment containing the EGFP3 expression unit was ligated to the pYEX-BX vector fragment using DNA Ligation Kit ver. 2, and *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pYEX+PHSP12+EGFP3+TCYC1 was prepared from this transformant.

A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with each of these 3 types of plasmids. The obtained transformant was inoculated into a synthetic medium containing no uracil in the same manner as in Example 6, followed by performing a shake culture at 30°C. In the case of the plasmid pYEX+PHSP12+EGFP3+TCYC1, however, since it had a weak leucine synthetase gene, an experiment wherein a medium formed by removing leucine from the above synthetic medium was used was also carried out. Culture, sampling, preparation of RNA, preparation of a protein, Northern blotting analysis, and SDS-PAGE analysis were all carried out in the same manner as in Example 6.

The middle and lower cases in Figure 19 show the amount of EGFP mRNA over time, obtained when the culture temperature for yeast transformed with these plasmids was decreased from 30°C to 10°C. The lower case in Figure 19 also shows the effects of removing leucine from the culture solution on the amount of the EGFP mRNA in the case of pYEX-BX.

As a result of Northern blotting analysis, in all cases of yeast transformed with 3 types of plasmids each having a different replication origin and a different selective marker (all of which comprised a DNA fragment having an HSP12 cold-inducible promoter function), the level of EGFP mRNA was increased by a low temperature treatment (10°C).

Figure 20 shows the amount of an EGFP protein over time, obtained when the culture temperature for yeast transformed with the plasmids shown in Figure 19 was decreased from

30°C to 10°C. The figure also shows the effects of removing leucine from the culture solution on the amount of the EGFP protein in the case of pYEX-BX.

As shown in Figure 20, when the expression level of the EGFP protein in yeast transformed with each of 2 types of plasmids pUG35+PHSP12+MCS and pYEX+PHSP12+EGFP3+TCYC1 was examined by SDS-PAGE analysis, it was found that the level of the EGFP protein was increased by a low temperature treatment (10°C) in both cases, and that a large amount of EGFP could be produced. In particular, the use of a plasmid having a leu2-d marker and a medium containing no leucine resulted in a significant production amount.

From the above studies, it was found that an expression plasmid comprising a DNA fragment having a cold-inducible promoter function enables cold-inducible production of a protein, regardless of a replication origin and a selective marker. On the other hand, it was also found that selection of such a replication origin or marker may lead to an increase in the production amount.

Subsequently, protein expression was carried out using different types of yeast strains of *Saccharomyces cerevisiae*. As such different types of yeast strains, YPH499, YPH501 (purchased from Stratagene), SHY3, KK4 (provided from Dr. Ryo Sato, an emeritus professor of Osaka University), EGY48 (purchased from Takara), and BY4741, BY4742 and BY4743 (purchased from Research Genetics) were used. These yeast strains were transformed with an expression plasmid pYEX+PHSP12+EGFP3+TCYC1. Each transformant was allowed to grow in a synthetic medium, to which necessary amino acids were added except for uracil, in the same manner as in Example 3. Thus, an intracellular protein was prepared, and analyzed by SDS-PAGE. Figure 21 shows the amount of an EGFP protein obtained when the culture temperature was decreased from 30°C to 10°C for various strains transformed with pYEX+PHSP12+EGFP3+TCYC1.

Expression of EGFP was observed in all the yeast strains. Thus, it was found that the DNA fragment having an HSP12 cold-inducible promoter function acts regardless of the type of yeast strain. In particular, when EGY48 strain or BY4743 strain was used, a high production amount of EGFP was obtained.

Example 8 Comparison of expression vector containing DNA fragment having cold-inducible promoter function with existing expression vectors in terms of expression level

pYES2 containing a galactose-inducible GAL1 promoter, pYEX-BX containing a heavy metal-inducible CUP1 promoter, and an expression plasmid pYEX+PHSP12+EGFP3+TCYC1 containing the aforementioned DNA fragment having an HSP12 cold-inducible promoter function (hereinafter referred to as pLTex221+EGFP3), were compared to one another under each recommended inducible conditions, in terms of the expression level of EGFP. The plasmid pYES2+EGFP3 produced in Example 7 was used as pYES2 containing EGFP. pYEX-BX containing EGFP was prepared as follows. The above plasmid pYES2+EGFP3 was cleaved with *Bam*HI and *Xho*I, and the cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate and recover EGFP3 ORF with a size of approximately 780 bp. On the other hand, pYEX-BX was cleaved with *Sal*I and *Bam*HI. The obtained EGFP3 ORF was ligated to pYEX-BX using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pYEX-BX+EGFP3 was prepared from this transformant. A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with each of these 3 types of plasmids (pYES2+EGFP3, pYEX-BX+EGFP3, and pLTex221+EGFP3). Culture, sampling, preparation of a protein, and SDS-PAGE analysis were carried out on the obtained transformant in the same manner as in Example 6. Figure 22 shows the results of SDS-PAGE analysis.

As shown in Figure 22, the expression vector pLTex221+EGFP3 containing a DNA fragment having an HSP12 cold-inducible promoter function produced a larger amount of EGFP3 than those of pYES2+EGFP3 and pYEX-BX+EGFP3. From these results, it became clear that an expression vector containing a DNA fragment having an HSP12 cold-inducible promoter function is more excellent than the existing expression vectors.

Example 9 Cold-inducible conditions applied in case of using expression vector containing DNA fragment having cold-inducible promoter function

Using a yeast strain *Saccharomyces cerevisiae* YPH500 transformed with the plasmid pYEX+PHSP12+EGFP3+TCYC1 (pLTex221+EGFP3) produced in Example 7, cold-inducible conditions were studied. The present transformed yeast was subjected to culture, sampling, preparation of a protein, and SDS-PAGE analysis by the same methods as in Example 8. Exposure to a low temperature was carried out at 4°C, 10°C, and 20°C, and sampling was carried out at 0, 6, 12, 24, 48, 72, and 96 hours after initiation of the low temperature treatment. Figure 23 shows the results of SDS-PAGE analysis.

As shown in Figure 23, production of an EGFP protein was observed in all the cases of the temperatures of 4°C, 10°C, and 20°C. From these results, it was found that using a DNA fragment having an HSP12 cold-inducible promoter function, cold-inducible production of protein can be carried out by a low temperature treatment, not only at 10°C, but also at 4°C or 20°C.

Example 10 Production of proteins using DNA fragment having cold-inducible promoter function in yeasts other than *Saccharomyces cerevisiae*

In order to examine whether or not a DNA fragment having a cold-inducible promoter function acts in yeasts other than *Saccharomyces cerevisiae*, a DNA fragment having an HSP12 cold-inducible promoter function and EGFP3 ORF were introduced into methylotrophic yeast *Pichia pastoris*.

First, pUG35+PHSP12+MCS produced in Example 7 was cleaved with *Bam*HI and *Kpn*I. The cleaved portion was then subjected to agarose gel electrophoresis, so as to fractionate and recover an approx. 1.7-kb DNA fragment comprising a DNA fragment having an HSP12 cold-inducible promoter function, EGFP3 ORF, and a CYC1 terminator. On the other hand, a plasmid pPICZ-B (purchased from Invitrogen) used for *Pichia pastoris* was cleaved with *Bam*HI and *Kpn*I. Thereafter, the cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate and recover an approx. 3.0-kb plasmid main body excluding an AOX1 terminator. The above DNA fragment was ligated to the plasmid

pPICZ-B excluding the AOX1 terminator using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pPICZ+PHSP12+EGFP3+TCYC1 was prepared from this transformant. A *Pichia pastoris* GS115 strain was transformed with this plasmid pPICZ+PHSP12+EGFP3+TCYC1 in accordance with the manual attached with Easy Select Pichia Expression Kit (Invitrogen). Subsequently, a stain resistant to 4 mg/ml Zeocin was selected. The obtained transformant was inoculated into a YPED medium, and it was then cultured at 30°C until the absorbance at 600 nm became 2.2. Thereafter, the culture temperature was decreased to 10°C by the same method as in Example 6, and sampling was carried out at 3 days and 10 days after the low temperature treatment. Preparation of a protein and Western blotting analysis were carried out by the same methods as in Example 6. Figure 24 shows the results of Western blotting analysis. Figure 24 shows the expression of an EGFP protein observed at 3 days and 10 days after the culture temperature was decreased from 30°C to 10°C, after the transformant *Pichia pastoris* had first been cultured at 30°C.

As shown in Figure 24, the EGFP protein was inducibly produced by decreasing the culture temperature for methylotrophic yeast *Pichia pastoris*. From these results, it was found that the cold-inducible expression with a DNA fragment having an HSP12 cold-inducible promoter function can be carried out not only in *Saccharomyces cerevisiae* but also in other types of yeasts.

Example 11 Expression of proteins other than EGFP protein using DNA fragment having cold-inducible promoter function

The possibility of expression of proteins other than the EGFP protein using a DNA fragment having a cold-inducible promoter function was confirmed as follows. Specifically, using a DNA fragment having an HSP12 cold-inducible promoter function, cDNA of an antifreeze protein RD3 (J. Biol. Chem. 276, 1304-1310 (2001)) was ligated downstream of the

aforementioned promoter. Thereafter, expression of the protein was confirmed by Western blotting analysis. It is to be noted that the RD3 protein became insolubilized, when it was allowed to express at 37°C in an expression system using *Escherichia coli* as a host.

An expression plasmid for RD3 was produced as follows. First, a plasmid pET20b/RD3 containing RD3 ORF (provided from Dr. Yoshiyuki Nishimiya of the National Institute of Advanced Industrial Science and Technology) was cleaved with *Nde*I and *Eco*RI, and the cleaved portion was then blunt-ended with DNA Blunting Kit. The resultant product was then subjected to agarose gel electrophoresis, so as to fractionate and recover a DNA fragment containing RD3 ORF (approximately 400 bp). On the other hand, the plasmid pUG35-MET25 produced in Example 3 was cleaved with *Hpa*I and *Mlu*I. The cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate a DNA fragment and to recover a vector fragment with a size of approximately 5.4 kb. Likewise, the plasmid pYES2+EGFP3 produced in Example 7 was cleaved with *Hpa*I and *Mlu*I. The cleaved portion was subjected to agarose gel electrophoresis, so as to fractionate and recover a fragment with a size of approximately 450 bp. The obtained vector fragment was ligated to the approx. 450-bp fragment using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the thus ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes, a transformant containing a plasmid of interest was identified. A plasmid pUG35-MET25+MCS of interest was prepared from this transformant. This plasmid pUG35-MET25+MCS was cleaved with *Eco*RI and *Not*I. The cleaved portion was then blunt-ended with DNA Blunting Kit, followed by performing dephosphorization with bacterial alkaline phosphatase. Thereafter, the resultant product was subjected to agarose gel electrophoresis, so as to recover a vector fragment (approximately 5.1 kb). The above DNA fragment containing RD3 ORF was ligated to the above vector fragment using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant

containing a plasmid of interest was identified. A plasmid pUG35-MET25+MCS+RD3 having RD3 ORF was prepared from this transformant.

Subsequently, a plasmid containing a DNA fragment having an HSP12 cold-inducible promoter function was produced. First, a DNA fragment having an HSP12 cold-inducible promoter function was amplified by PCR according to the method described in Example 4. The termini thereof were phosphorylated with T4 polynucleotide kinase, and fractionation of DNA fragments was then carried out by agarose gel electrophoresis, so as to recover a DNA fragment (approximately 610 bp) having an HSP12 cold-inducible promoter function. On the other hand, pUG35-MET25+MCS+RD3 was cleaved with *Spe*I. The cleaved portion was then blunt-ended with DNA Blunting Kit, followed by performing dephosphorization with bacterial alkaline phosphatase. The above DNA fragment having an HSP12 cold-inducible promoter function was ligated to the above vector fragment using DNA Ligation Kit ver. 2. Thereafter, *Escherichia coli* DH5α was transformed with the ligated product. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant containing a plasmid of interest was identified. Thus, an expression plasmid containing a DNA fragment having an HSP12 cold-inducible promoter function was finally prepared from this transformant.

Moreover, a plasmid containing a TDH3 promoter was produced as follows. First, a yeast expression vector pG-3 containing a TDH3 promoter was cleaved with *Bam*HI and *Hind*III. The cleaved portion was then blunt-ended with DNA Blunting Kit, and fractionation of DNA fragments was carried out by agarose gel electrophoresis, so as to recover a fragment containing a TDH3 promoter (approximately 660 bp). The obtained DNA fragment was inserted into the *Spe*I site of pUG35-MET25+MCS+RD3 by the same method as described above. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant containing a plasmid having a structure of interest was selected. Thus, an expression plasmid containing a TDH3 promoter was finally prepared.

These two types of plasmids have the same structure other than their promoters. A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with each of these 2 types of

plasmids. The obtained transformant was inoculated into a synthetic medium containing no uracil, followed by performing a shake culture at 30°C. With regard to yeast transformed with an expression plasmid containing a TDH3 promoter, a culture solution thereof was recovered at the time when the absorbance at 600 nm became 0.7. With regard to yeast transformed with an expression plasmid containing a DNA fragment having an HSP12 cold-inducible promoter function, culture and sampling were carried out by the same experimental methods as in Example 6 with exception that a low temperature treatment was initiated at the time when the absorbance at 600 nm became 1.0. In Western blotting analysis, a 5000 times diluted anti-RD3-Nl antibody was used (which was an antibody recognizing the subunit of RD3, which was produced by Hokudo Co., Ltd., according to our request). Figure 25 shows the results of Western blotting analysis showing the expression level of the RD3 protein obtained when the transformed yeast was cultured, while decreasing the temperature from 30°C to 10°C, or at 30°C. From these results, it was found that when the RD3 proteins that are insolubilized in an *Escherichia coli* expression system are inducibly produced at 10°C using a DNA fragment having an HSP12 cold-inducible promoter function, almost the proteins are produced as soluble proteins. It could also be confirmed that the use of the DNA fragment having an HSP12 cold-inducible promoter function enables production of a larger amount of protein than the case where the protein is produced at 30°C using the existing TDH3 promoter.

Subsequently, as in the case of RD3, ECFP and DsRed were allowed to express. In order to produce ECFP and DsRed not as fusion proteins but as natural proteins, each ORF region encoding the natural proteins from pECFP and pDsRed-Express (both of which were purchased from Clontech) was amplified by PCR, and each amplified product was then introduced into expression vectors pTrc99A (purchased from Pharmacia). *Escherichia coli* was transformed with each of these expression plasmids, but no fluorescence derived from a fluorescent protein was observed.

First, a cold-inducible expression vector pLTex321 having a multicloning site was constructed. pUG35-MET25+MCS was cleaved with *Cla*I and *Xho*I. The cleaved portion was then subjected to agarose gel electrophoresis, so as to recover a vector fragment

(approximately 5.1 kbp). In order to circularize this vector fragment, the following oligo DNAs were synthesized and used as linkers.

MCS linker F: CCGCTCGAGCGGCCGCGAGCTCGTCGACATCGATGG (SEQ ID NO: 27)

MCS linker R: CCATCGATGTCGACGAGCTCGCGGCCGCTCGAGCGG (SEQ ID NO: 28)

The linker DNAs contain a restriction site of *Xho*I-*Not*I-*Sac*I-*Sal*I-*Cla*I. Both oligo DNAs were annealed, and both termini of each of the linker DNAs were cleaved with *Xho*I and *Cla*I. The above vector fragment was ligated to the linker DNA using DNA Ligation Kit ver. 2. Thereafter, the ligated product was introduced into *Escherichia coli* DH5α. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant containing a plasmid of interest was identified. A plasmid of interest was prepared from this transformant.

The obtained plasmid was further cleaved with *Spe*I and *Bam*HI, and the cleaved portion was subjected to agarose gel electrophoresis, so as to recover a vector fragment (approximately 5.1 kb). In order to introduce a DNA fragment having an HSP12 cold-inducible promoter function into the obtained vector fragment, a DNA fragment having an HSP12 cold-inducible promoter function and containing a *Spe*I recognition sequence and a *Bam*HI recognition sequence was amplified by PCR using the primers indicated below. PCR was carried out under the same conditions as in amplification of an HSP12 fragment in Example 2.

-610-HSP12 IGR SpeI F: GGACTAGTGATCCCACTAACGGCCCAG (SEQ ID NO: 29)

-610-HSP12 IGR BamHI R: CGGGATCCTGTTGTATTTAGTTTTTTTTGTTTTGAG (SEQ ID NO: 30)

Thereafter, the amplified product was cleaved with *Spe*I and *Bam*HI, followed by fractionation by agarose gel electrophoresis, so as to recover a DNA fragment (approximately 600 bp) having an HSP12 cold-inducible promoter function.

The above vector fragment was ligated to the DNA fragment having an HSP12 cold-inducible promoter function using DNA Ligation Kit ver. 2. Thereafter, the ligated product was introduced into *Escherichia coli* DH5α. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a plasmid of interest was prepared.

The obtained plasmid was cleaved with *Spe*I and *Kpn*I, and the cleaved portion was subjected to agarose gel electrophoresis, so as to recover a DNA fragment (approximately 1 kb) containing the DNA fragment having an HSP12 cold-inducible promoter function, a multicloning site, and a CYC1 terminator. Likewise, a pYEX-BX expression vector was cleaved with *Spe*I and *Kpn*I, and the cleaved portion was subjected to agarose gel electrophoresis, so as to recover a vector fragment (approximately 6.4 kb). The above DNA fragment containing the DNA fragment having an HSP12 cold-inducible promoter function, a multicloning site, and a CYC1 terminator was ligated to the above vector fragment using DNA Ligation Kit ver. 2. Thereafter, the ligated product was introduced into *Escherichia coli* DH5α. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, an expression vector pLTex321 was prepared.

On the other hand, an expression plasmid of ECFP was produced as follows. First, ECFP ORF was prepared from a plasmid pECFP by PCR.

The sequences of the used primers were as follows.

BAMCFP1: AAAAGGATCCAAAAAAATGGTGAGCAAGGGCGAGGAG (SEQ ID NO: 31)

HNDCFP2: TTTTAAGCTTTTACTTGTACAGCTCGTCCAT (SEQ ID NO: 32)

BAMCFP1 comprises, in the order from the 5'-terminal side, 4 A bases, a *Bam*HI recognition sequence, 6 A bases, and the downstream 21-bp portion from the initiation codon of ECFP ORF in pECFP. HNDCFP2 comprises, in the order from the 5'-terminal side, 4 T bases, a *Hind*III recognition sequence, and a sequence complementary to the upstream 21 bases from the termination codon of ECFP ORF.

PCR was carried out using 50 μl of a reaction solution containing 1ng pECFP, 300 nM each primer, 200 μM dNTP, 1 mM $MgSO_4$, and a 1 x PCR buffer used for KOD -Plus- (Toyobo Co., Ltd.) and 1U KOD -Plus- DNA polymerase, under conditions consisting of: a first step of 94°C, 2 minutes; and a second step of 30 cycles consisting of 94°C, 15 seconds (denaturation), 45°C, 30 seconds (annealing), and 68°C, 1 minute (elongation). Thereafter, the amplified DNA was cleaved with *Bam*HI and *Hind*III. On the other hand, the above produced expression vector pLTex321 was cleaved with *Bam*HI and *Hind*III. The ECFP ORF amplified by the above PCR was ligated to the pLTex321 vector fragment using Ligation High (Toyobo Co., Ltd.) Thereafter, the ligated product was introduced into *Escherichia coli* DH5α. The obtained transformant was cultured overnight, and a plasmid was then extracted using QuantumPrep Plasmid MiniPrep kit. Based on a cleavage pattern made by restriction enzymes and sequence analysis, a transformant containing a plasmid of interest was identified. A plasmid pLTex321+ECFP having ECFP was prepared from this transformant.

With regard to DsRed, pLTex321+DsRed was produced under the same conditions as for the above ECFP with exception that the primers indicated below were used and that pDsRed-Express was used as a template for PCR.

The sequences of the used primers are as follows.

BAMRED1: AAAAGGATCCAAAAAAATGGCCTCCTCCGAGGACGTC (SEQ ID NO: 33)

HNDRED2: AAAAAAGCTTCTACAGGAACAGGTGGTGGCG (SEQ ID NO: 34)

BAMRED1 comprises, in the order from the 5'-terminal side, 4 A bases, a *Bam*HI recognition sequence, 6 A bases, and the downstream 21-bp portion from the initiation codon of DsRed ORF in pDsRed-Express. HNDRED2 comprises, in the order from the 5'-terminal side, 4 A bases, a *Hind*III recognition sequence, and a sequence complementary to the upstream 21 bases from the termination codon of DsRed ORF.

A yeast strain *Saccharomyces cerevisiae* YPH500 was transformed with each of these 2 types of plasmids thus produced. The obtained transformant was inoculated into a synthetic medium containing neither uracil nor leucine, followed by performing a shake culture at 30°C. At the time when the absorbance at 600 nm became approximately 0.9, the culture product

was subjected to a low temperature treatment at 10°C. Then, culture was continued at 10°C for 24 hours. The expression of a fluorescent protein was confirmed with fluorescence under a UV lamp (356 nm). The results are shown in Figure 26. As shown in Figure 26, in both cases of ECFP expression yeast and DsRed expression yeast, a strong fluorescence due to the produced fluorescent protein was observed.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

Industrial Applicability

The present invention provides a DNA fragment having a cold-inducible promoter function of yeast. The DNA fragment of the present invention is useful in that it can be used in production of a protein and in regulation of production of RNA at a low temperature. The present invention enables the development of a novel protein production system utilizing advantages of a low temperature, such as production of a protein, the expression of which has previously been difficult. In addition, it is considered that the present invention promotes clarification of cold inducibility in terms of molecular mechanism.